IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Ivins *et al.*
Application No. 10/533,634
Filed: April 29, 2005
Confirmation No. 5041
For: METHOD OF PREVENTING INFECTIONS FROM BIOTERRORISM AGENTS WITH IMMUNOSTIMULATORY CPG OLIGONUCLEOTIDES
Examiner: Emily M. Le
Art Unit: 1648
Attorney Reference No. 4239-67021-06

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DECLARATION UNDER 37 C.F.R. § 1.132

1. I, Dennis M. Klinman, M.D., Ph.D., am an inventor named in the above-referenced patent application. I am currently Head of the Immune Modulation Group at the National Cancer Institute of the National Institutes of Health (NIH). I have been employed by the Food and Drug Administration or the NIH continuously since 1983 and have conducted research in the fields of virology, immunology and vaccine development over that 25 year period. I have published more than 250 scientific articles in the fields of virology, immunology and vaccine development. A copy of my current curriculum vitae is submitted herewith.

2. I have read and understood the above-referenced patent application, including the pending claims, and the Office action dated July 31, 2008.

3. It is my understanding that in the Office action dated July 31, 2008, claims 37, 38, 40, 41, 50, 52-57 and 61-64 were rejected as allegedly obvious over Ivins *et al.* (*Eur. J. Epidemiol.* 4(1):12-19, 1988), in view of Verthelyi *et al.* (*J. Immunol.* 168:1659-1663, 2002) and Jones *et al.*

(*Vaccine* 17:3065-3071, 1999). It is also my understanding that it is alleged that it would have been obvious to use the CpG oligonucleotide adjuvant of Jones *et al.* with the anthrax vaccine of Ivins *et al.* in view of the teaching of Verthelyi *et al.* that CpG oligonucleotides can be used as adjuvants in primates.

4. Based on my experience in the field of vaccine development, it is not possible to predict whether CpG oligonucleotides of the K-type or D-type class will enhance the immunogenicity of a specific vaccine without empirical data. This is evidenced by Verthelyi *et al.* 2002, cited by the Office, which teaches that D-type oligonucleotides, but not K-type oligonucleotides, are effective when used with a heat killed *Leishmania* vaccine (HKLV). Verthelyi *et al.* describe work performed in my laboratory in which K-type and D-type CpG oligonucleotides were tested as adjuvants for HKLV. As shown in Figure 5 of Verthelyi *et al.*, D-type CpG oligonucleotide effectively enhanced the immune response elicited by HKLV, as evidenced by a decrease in lesion size following challenge with metacyclic promastigotes. In contrast, K-type CpG oligonucleotide did not significantly alter lesion size relative to vaccine alone. Of note, our empirical studies showed that K-type oligonucleotides but not D-type oligonucleotides were highly effective adjuvants when used in combination with AVA (the licensed anthrax vaccine), a result that directly contrasts with their relative activity when combined with HKLV.

5. In addition, my laboratory conducted studies in which a K-type CpG oligonucleotide was tested as an adjuvant for whole, inactivated influenza virus (heat killed PR8 influenza virus HKF). In these studies, we compared the magnitude of the IgG anti-influenza antibody response and protection conferred to mice treated with HKF alone or in combination with 50 μg/ml K-type CpG oligonucleotide or 50 μg of the lipid adjuvant monophosphoryl lipid A (MPL). The results showed that adding MPL significantly improved (more than doubled) the serum anti-influenza antibody response ($p < 0.02$) and improved survival (100% survival, $p < 0.05$). In contrast, the K-type CpG oligonucleotide did not significantly increase anti-influenza antibody levels or survival ($p > 0.35$ for both parameters). This result is in stark contrast to the results obtained using a K-type CpG oligonucleotide (CpG 7909; SEQ ID NO: 200) in combination with the anthrax vaccine AVA in

macaques. As described in the specification, administration of AVA + CpG 7909 resulted in a 17-fold increase in neutralizing antibody titer relative to administration of AVA alone. Furthermore, serum from macaques vaccinated with AVA + CpG 7909 significantly increased passive protection to mice challenged with *Bacillus anthracis*, relative to serum obtained from macaques vaccinated with AVA alone.

6. These studies demonstrate that it is not possible to predict if a particular class of CpG oligonucleotide will function as an effective adjuvant for a vaccine without experimental testing. Therefore, one of skill in the art would not have been able to predict that a CpG oligonucleotide having the nucleotide sequence of SEQ ID NO: 200 would enhance the immunogenicity of an anthrax vaccine. Furthermore, given the findings described in Verthelyi *et al.* and other studies performed in my laboratory on influenza vaccines, it would not have been expected that a K-type oligonucleotide, such as an oligonucleotide having the nucleotide sequence of SEQ ID NO: 200, would enhance the immunogenicity of an anthrax vaccine, such as AVA.

7. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. § 1001, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: OCT 30, 2008

Dennis M. Klinman, M.D., Ph.D.

CURRICULUM VITAE
Dennis M. Klinman, MD/PhD

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Work Address:
Bldg 567 Rm 205
Center for Cancer Research, National Cancer Institute, Frederick, MD 21702
(301) 228-4265 (Work)
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E mail: klinmand@mail.nih.gov

Date/Place of Birth:
October 9, 1954; Philadelphia, PA

Marital Status:
Married to Dr. Susan Leitman-Klinman
Three daughters

Education:

1976	BS, Saint Joseph's University
1978	Graduate Member, Cambridge University
1982	MD/PhD, University of Pennsylvania
1987	Rheumatology Fellowship, NIAMS, NIH

Postgraduate Experience:

1982-83	Medical Internship, The Faulkner Hospital, Boston, MA
1983-89	Medical and Senior Staff Fellow, National Institutes of Health, Bethesda, MD
1989-91	Medical Officer, Center for Biologics Evaluation and Research, FDA, Bethesda, MD
1991-93	Tenured Senior Investigator, Division of Viral Products, CBER/FDA, Bethesda, MD.
1993 - 2007	Chief, Retroviral Immunology Section, Division of Viral Products, CBER/FDA, Bethesda, MD.
2007 -	Chief, Immune Modulation Section, Laboratory of Experimental Immunology, NCI/NIH, Frederick, MD.

Grants: (since 2000)

Interagency Agreement, NIAID	$ 25,000, 2000
Research Grant, USAMRIID	$ 202,000, 2000
National Vaccine Program,	$ 79,000, 2000
CBER Counter-BioT Program	$ 434,000, 2000

Patent income	$ 175,000, 2000
CRADA - Coley Pharm	$ 250,000, 2001
National Vaccine Program,	$ 76,000, 2001
Research Grant, USAMRAA	$ 120,000, 2002
CBER Counter-BioT Program	$ 92,000, 2002
CRADA - Coley Pharm	$ 250,000, 2002
Patent income	$ 95,000, 2002
CBER Counter-BioT Program	$ 40,000, 2003
Research Grant, USAMRAA	$ 429,000, 2003
Research Grant, DARPA	$ 257,000, 2003
Patent income	$ 143,000, 2003
National Vaccine Program	$ 79,000, 2004
Patent income	$ 87,000, 2004
National Vaccine Program	$ 223,000, 2005
Patent income	$1,050,000, 2005
Defense Threat Reduction Agency	$ 225,000, 2006
National Vaccine Program	$ 180,000, 2006
Defense Threat Reduction Agency	$ 225,000, 2007
Defense Threat Reduction Agency	$ 215,000, 2008

Honors:

University Scholar, St. Joseph's University, 1976
Presidential Scholar, St. Joseph's University, 1972 - 1976
Summa Cum Laude, St. Joseph's University, 1976
Medical Scientist Training Program, Univ of Penna, 1976 - 1982
Baldwin Lucke Prize in Experimental Medicine, 1981
R.G. Williams Prize in Basic Medical Science, 1982
Regional Rheumatology Award, Amer. Rheumatology Association, 1987
Senior Rheumatology Award, ARA, 1987
Outstanding Achievement Award in the Biological Sciences, Washington Academy of Science, 1988
Public Health Service Outstanding Service Medal, 1992
Member, American Society for Clinical Investigation, 1992
Public Health Service Outstanding Unit Citation, 1994
Kunkel Award for Outstanding Research in the field of Rheumatology, American College of Rheumatology, 1994
Outstanding Scientist Award, Center for Biologics Evaluation and Research, FDA 1995
Outstanding Scientist of the Year Award, Food and Drug Administration, 1995
Public Health Service Meritorious Service Medal, 1997
Arthritis Foundation “Hero”, 2001
Public Health Service Distinguished Service Medal, 2004

Counter-Bioterrorism Activities

Security Clearance: **Secret**

Chairman, CBER Bioterrorism Response Working Group, 1998 - 2002.
Organizer, CBER-USAMRIID Symposium on Bioterrorism Threats, Oct 1999.
Participant, AMEDD Technology Workshop, Department of the Army, McLean, VA 1999.
Speaker, "Current capabilities of biowarfare pathogens", SAG 2015 Threat Assessment Conference, National Intelligence Office, Mclean, VA 2000.
Speaker, "Use of CpG Oligonucleotides to prevent infection by biowarfare agents", USAMRIID, Ft. Detrick, MD, 2000
Participant, Biological Terrorism and Technology-Based Threat Assessment Workshop, Institute for Security Technology Studies, Dartmouth College, NH, 2000.
Participant, Future Technology Seminar 2000, US Army Training and Doctrine Command, Ft. Monroe, VA, 2000.
Speaker, "Induction of protective immune responses by CpG oligodeoxy-nucleotides", Biodefense: Research, Technologies and Applications, McLean, VA 2002.
Participant, Biosecurity 2002 Conference, Las Vegas, NV 2002.
Participant, Unconventional Pathogen Countermeasures conference, Galveston, Tx, 2003
Member, Scientific Advisory Committee, Agile Vaccine Program, WRAIR
Lecture, "Use of CpG Oligonucleotides for Biodefense", University of Texas Medical Branch, Galveston, Tx, 2003
Member, Information Panel on Advances in Biological Threat Reduction, Miter Corporation, Vienna, VA, 2004
FDA representative, Molecular Vaccine Interagency Working Group, White House Conference Center, 2004
Speaker, "Use of CpG DNA and other TLR Ligands to Modulate Host Defense", American Academy of Allergy, Asthma and Immunology Annual Meeting, San Francisco, CA, 2004
Speaker, "The Application of Immunotherapies to Biodefense", Biodefense Vaccines, Therapeutics and Diagnostics, Washington DC, 2004.
Speaker, "The Use of CpG Oligonucleotides to Modulate Host Defense", First National Congress on Public Health Readiness, Washington DC, 2004.

Scientific Reviewer for the following journals:

AIDS
American Journal of Pathology
Arthritis and Rheumatism
Blood
Cancer Research
Clinical Immunology and Immunopathology
Human Gene Therapy

Immunopharmacology
Immunity
Journal of Acquired Immune Deficiency Syndrome
Journal of Clinical Immunology
Journal of Clinical Investigation
Journal of Experimental Medicine
Journal of Immunology
Journal of Immunological Methods
Journal of Molecular Immunology
Journal of Rheumatology
Journal of Virology
Nature Biotechnology
Proceedings of the National Academy of Sciences, USA
Scandinavian Journal of Immunology
Vaccine
Viral Immunology

Scientific Advisor or Reviewer for the following organizations:

American Rheumatology Association: Grants Review
American College of Rheumatology: Abstract Selection
Arthritis Council of Great Britain
Arthritis Council of Canada
Booz Allen and Hamilton
Broad Foundation
Defense Sciences Research Council, Universal Medical Countermeasures
Defense Threat Reduction Agency
Dept. of Veterans Affairs - Career Development Grants
Hughes Medical Research Institute
National Intelligence Office, Science and Technology Expert

Partnership (STEP)
NIAID: Extramural Program Project Grants Review
NIAID Malaria Vaccine Initiative
NCI: Extramural Program Project Grants Review
Office of Woman's Health
Sabin Vaccine Institute
Scientific Advisory Committee, Agile Vaccine Program, WRAIR
United States - Israel Binational Science Foundation

Teaching Appointments:

Lecturer, Foundation for Advanced Education in the Sciences, NIH. Course Title: "Seminars in Immunology" (since 1991).
Lecturer, FAES, NIH. Course Title: "Cellular and Molecular Mechanisms of Immunity".
Lecturer, Center for Drug Evaluation and Research Staff College,

FDA. Course Title: “Applied Immunology” (Since 1993).

Membership in Professional Societies:

American Association of Immunologists
American Association for the Advancement of Science
American Society for Clinical Investigation
New York Academy of Sciences

Selected Professional Presentations (from over 200 presentations)

Speaker, Conference on Immune Intervention, Montpelier, France, 1986.
Speaker, "B cell activation in Autoimmune Disease", Yokohama City University, Japan, 1988.
Speaker, "B cell activation in SLE", Combined Clinical Conference on Systemic Lupus Erythematosus, (Grand Rounds) NIH, 1991.
Chairman, Session on Murine Models of SLE, UCLA Symposium on Self Reactivity and its Regulation, Keystone, CO., 1991.
Speaker, "Cell line issues related to specific expression vectors", Fifth ABC International Biotechnology Meeting, Washington, DC. 1991.
Speaker, "Active and passive immunotherapy of HIV", VII International Conference on AIDS, Amsterdam, The Netherlands, 1992
Speaker, "Polyclonal B cell activation in Lupus", International Symposium on Immunobiology of Proteins and Peptides, Alberta, Canada, 1992.
Speaker, "Abnormalities in Lymphokine Production in HIV-Infected Patients", Yokohama City Medical School, Yokohama, Japan, 1994.
Speaker, "Alterations in the ratio of Th1 : Th2 type cytokine secreting cells in Pathologic States", Shimane Medical Univ., Shimane, Japan, 1994.
Chairman "Immunogenicity: Intended and Unintended", FDA Intramural Immunology Workshop, Rockville, MD, 1995
Co-Chair, "Immunity Induced by DNA Vaccines" American Society for Microbiology, New Orleans, LA, 1996.
Speaker, "The Scientific Future of Genetic Immunizations", American Academy of Microbiology, Aruba, Dutch Antilles, 1996.
Speaker, "DNAse: Novel Therapy for the treatment of SLE”, American College of Rheumatology National Meeting, Orlando, Fl, 1996.
Speaker, “Contribution of CpG motifs to the immunogenicity of DNA vaccines”, Mt. Sinai Medical Center, New York, NY, 1997.
Speaker, “Tolerance Induction by DNA Vaccines”, Gordon Conference on Genetic Vaccination, Plymouth, New Hampshire, 1997.
Speaker, “Safety of DNA Vaccines”, NIBSC, Hertfordshire, England, 1997.
Speaker, “Immunotherapeutic Potential of CpG Motifs”, International Working Group on DNA Vaccines, London, England, 1998.
Chairman, Session on the “Mechanism of Action of DNA Vaccines”, Keystone Symposium on DNA Vaccines, Snowbird, Utah,1999

Speaker, "DNA Vaccines and Autoimmunity", 5th International Symposium on Autoimmunity, Limmisol, Cyprus, 1999.
Speaker, "CpG motifs stimulate human cells", 1st International Symposium on CpG Oligonucleotides, Schloss Elmau, Germany, 1999.
Speaker, "Safety of DNA Vaccines", International Working Group on DNA Vaccines, Munich, Germany, 1999.
Speaker and Co-Chairman, "Cellular Uptake and Activation by CpG Oligonucleotides" Fourth NIH Symposium on Therapeutic Oligonucleotides, Bethesda, MD, 1999.
Speaker, "Immunogenicity of DNA vaccines", Millennium Conference on Nucleic Acid Therapeutics, Clearwater Beach, Fl, 2000.
Speaker, "Capacity of DNA Vaccines to Induce Autoimmunity", WHO International Workshop on Nucleic Acid Vaccines, Geneva, Switzerland, 2000.
Speaker, "Therapeutic Applications of CpG Oligonucleotides", Yokohama City University Medical School, Yokohama, Japan, 2000.
Speaker, "Effect of Bacterial DNA on the Development of Autoimmune Disease", Japanese International Rheumatology Meeting, Yokohama, Japan, 2000.
Speaker, "Activity of Immunostimulatory and Immunosuppressive Motifs in Mammalian and Bacterial DNA", Japanese National Institutes of Health, Tokyo, Japan, 2000.
Speaker, "Safety of DNA Vaccines", Euregenethy Forum on Gene Therapy, Paris, France, 2000.
Speaker, "Immunostimulatory and immunosuppressive activity of DNA", LFKRI, New York, NY, 2001.
Speaker, "Immunoprotective activity of CpG Oligonucleotides", 2nd Annual Symposium on the Immunology of Infectious Disease, USUHS, Bethesda, MD, 2001.
Speaker, "CpG ODN induce protection against biothreat pathogens", 2nd International Symposium on CpG Oligos, Ameilia Island, FL, 2001.
Speaker, "CpG adjuvant activity", Vaccines of the future: from rational design to clinical development. Paris, France, 2001.
Speaker, "Role of CpG stimulatory and inhibitory motifs in innate and cognate immune responses", Keystone Symposium, Breckenridge, CO. 2002.
Speaker, "CpG Oligonucleotides as Immune Adjuvants", IMV 2002 Vaccine Conference, Prague, Czechoslovakia
Co-chairman and speaker, "Innate immunity Session", Trudeau Institute Symposium on Immunity to Viral Infections, Saranac Lake, NY, 2002.
Speaker, "Induction of protective immune responses by CpG oligodeoxy-nucleotides", Biodefense: Research, Technologies and Applications, McLean, VA 2002.
Speaker, "Suppressive Oligonucleotides regulate CpG Induced Immune

Activation", 6th NIH Symposium on Therapeutic Oligonucleotides. Bethesda, MD 2002.

Chairman, Workshop on "Evaluating Nucleic Acid Based Vaccines", 7th Symposium on Regulatory and Analytical Sciences, San Francisco, CA 2003.

Plenary Lecture, "Science Based Regulation of DNA Vaccines", 7th Symposium on Regulatory and Analytical Sciences, San Francisco, CA 2003.

Lecture, "Use of CpG Oligonucleotides for Biodefense", University of Texas Medical Branch, Galveston, Tx, 2003

Speaker, :"Protective Innate Immune Responses Elicited by CpG Nucleotides", FDA Science Forum, Washington, DC, 2003

Speaker, "Harnessing Immunostimulation CpG Oligonucleotides for BioDefense", The Emerging Role of Toll-like Receptors in Biology and Medicine, Boston University School of Medicine, Boston MA, 2003.

Speaker, "Immunomodulatory Activity of DNA", Keystone Symposium on Vaccines, Keystone, CO, 2004.

Speaker, "Use of CpG DNA and other TLR Ligands to Modulate Host Defense", American Academy of Allergy, Asthma and Immunology Annual Meeting, San Francisco, CA, 2004.

Lecture, "Therapeutic Applications of Immunomodulatory Oligonucleotides", Elkin Lecture Series, Winship Cancer Institute, Emory University, Atlanta, GA, 2004.

Lectures "Mode of Action of Immune Adjuvants" and "Safety of CpG-based Immune Adjuvants", World Health Organization Global Vaccine Safety Advisory Committee, Geneva, Switzerland, 2004.

Speaker "How CpG Adjuvants Interface with the Innate Immune System", Conference on Innate and Adaptive Immunity, Annecy, France, 2004.

Speaker, "The Use of CpG Oligonucleotides to Modulate Host Defense", First National Congress on Public Health Readiness, Washington DC, 2004.

Speaker, "Use of CpG Oligonucleotides as Vaccine Adjuvants", NIH Vaccine Working Group, Bethesda, MD 2005

Speaker, "Modulation of Disease Susceptibility through Activation and Suppression of the Innate Immune System", Society of Toxicological Pathology Annual Meeting, Washington DC, 2005.

Speaker, "Enhancement of Anthrax Vaccination in Primates by CpG ODN", Oligonucleotide Therapeutics Society Meeting, New York, NY, 2005.

Chairman, Session on "Innate Immunity and Antigen Presentation", NIH Immunology Retreat, Airlie, VA, 2005.

Speaker, "Innate Immune Modifiers: Activity and Safety as Vaccine Adjuvants", Medical Countermeasures and Innate Immunity, National Defense University, Washington DC, 2005.

Speaker, "Proposed Guidance on Prophylactic DNA Vaccines", 3rd International Symposium on DNA Vaccines, London, England, 2006.
Speaker, "Use of CpG oligonucleotides to improve host protection against infectious pathogens", Keystone Symposium on Viral Immunity, Steamboat Springs, CO, 2006.
Speaker, "CpG oligonucleotides: role in vaccine development", 9th Annual conference on Vaccine Research, Baltimore, MD, 2006.
Speaker, "Therapeutic applications of immunomodulatory oligonucleotides", Johns Hopkins University, Baltimore, MD, 2006.
Moderator, Session on "DNA Vaccine Electroporation" and Speaker "Insights into FDA regulation of DNA vaccines", DNA Vaccines 2007, Malaga, Spain, 2007.
Speaker, "Gene Activation Cascade Mediated by CpG Oligonucleotides", Microbiology Technical University of Munich, Munich, Germany, 2007.
Speaker, "Immunomodulatory Oligonucleotides: Impact on Immunity and Disease", Immunology Interest Group, NIH, Bethesda, MD, 2007.
Speaker, "CpG Oligonucleotides as Adjuvants for the Anthrax Vaccine", Systems Integration in Biodefense, Washington, DC, 2007.
Co-organizer and speaker, "Impact of CpG and Suppressive Oligonucleotides on Diseases associated with Inflammation", Cancer and Inflammation Symposium, Washington, DC, 2007.
Co-organizer and symposium chairman, Molecular Vaccine Development Symposium, Islamorada, FL, 2008.
Chairman and speaker, "Factors influencing DNA vaccine development", DNA Vaccines Forum 2008, London, England, 2008.
Speaker, "Advice to young investigators", NCI Fellows Retreat, Ocean City, MD, 2008.
Speaker, "Enhancement of AVA immunogenicity by CpG Oligonucleotides", 11th Annual conference on Vaccine research, Baltimore, MD, 2008.
Speaker, "Immunomodulatory activity of CpG ODN: opportunities for TLR based immune therapy to treat infectious diseases and cancer", Bilkent University, Ankara, Turkey, 2008 .
Speaker, "Immunosuppressive DNA to treat systemic inflammatory and autoimmune disease", GATA Medical School, Ankara, Turkey, 2008.

Patents (selected from over 150 patents in the area of CpG and suppressive ODN)

Immunomostimulatory Nucleic Acid Molecules. Patent No. 6,207,646 B1.

Use of CpG Oligodeoxynucleotides to Encourage Angiogenesis DHHS Reference No. E-328-01/0.

Immune Activation by Double-Stranded Polynucleotides. Application No. 09/151,612

Identification of DNA Sequence Motifs that Suppress the Immune Response to CpG DNA DHHS Reference No. E-218-01/0.

Use of Sterically Stabilized Cationic Liposomes to Efficiently Deliver CpG Oligonucleotides in vivo DHHS Reference No. E-215-01/0.

Novel Method for Rapidly Generating Mature Dendritic Cells from Peripheral Blood Monocytes and Myeloid Precursors DHHS Reference No. E-214-01/0

Method of Treating and Preventing Infections in Immunocompromised Subjects with Immunostimulatory CpG Oligonucleotides DHHS Reference No. E-153-02/0.

Methods for Treating and Preventing Infectious Disease Serial number 10/187,489.

Methods for Treating and Preventing Infectious Disease in Non-human Animals. Serial number 09/629,477.

Methods and Products for Treating HIV Infection. Serial No. 09/931,583.

Oligodeoxynucleotide and its use to Induce and Immune Response. DHHS Reference No. E-147-99/2

Method for Treating Inflammatory Arthropathies with Suppressors of CpG Oligonucleotides. DHHS Reference No. E-154-02/2.

Multiple CpG Oligodeoxynucleotides and their Use to Induce and Immune Response. DHHS Reference No. E-078-00/1.

Immunostimulatory RNA/DNA hybrid molecules. Pub #: US-2004-0052763-A1

BIBLIOGRAPHY

1) **Klinman DM** and Howard JC: Radioiodination of monoclonal antibodies. In: Monoclonal Antibodies, Ed. Kennett R. Plenum Press, NY p. 401, 1980.

2) **Klinman DM** and McKearn TJ: Dialyzable serum components support the growth of hybridoma cell lines in vitro. J. Immunol. Meth. 42:1, 1981.

3) **Klinman DM,** Smilek DE and McKearn TJ: Class I major histocompatibility gene products of the Brown Norway rat display two major antigenic regions. J. Immunol. 129:1204, 1982.

4) **Klinman DM:** Immune recognition of Class I Major Histocompatibility Antigens. Thesis. 1982.

5) **Klinman DM**: Analysis of non-dominant idiotypes during alloimmune responses. Cell. Immunol. 87:126, 1984.

6) Miller ML, Raveche ES, Laskin CA, **Klinman DM** and Steinberg AD: Genetic studies in NZB mice. VI. Association of autoimmune traits in recombinant inbred lines. J. Immunol. 133:1325, 1984.

7) Mountz JD, Mushinski JF, **Klinman DM** and Steinberg AD: Autoimmunity and increased c-myb transcription. Science 226:1087, 1984.

8) Hudgins CC, Steinberg RT, **Klinman DM**, Reeves JP and Steinberg AD: Studies on consomic mice bearing the Y chromosome of the BXSB mouse. J. Immunol. 134:3849, 1985.

9) Smith HR, Yaffe LJ, Chused TM, Raveche ES, **Klinman DM** and Steinberg AD: Analysis of B cell subpopulations: I. Relationships among splenic xid, Ly 1^+ and LyB 5^+ cells. Cell. Immunol. 92:190, 1985.

10) Steinberg AD, Triem KH, Smith HR, Laskin CA, Rosenberg YJ, **Klinman DM**, Mushinski JF and Mountz JD: Studies of the effects of Y chromosome factors on the expression of autoimmune disease. Ann. NY Acad Sci, 1985.

11) Mountz JD, Smith HR, **Klinman DM**, Mushinski JF and Steinberg AD: Modulation of c-myb oncogene transcription in autoimmune disease by cyclophosphamide. J. Immunol. 135:2417, 1985.

12) Steinberg AD, **Klinman DM**, Rosenberg YJ, Honda M, Santoro TJ, Reeve JP, Seldin MF, Mushinski JF and Mountz JD: Autoimmune lymphoproliferative disorders: Studies of pathogenesis and approaches to therapy. In: Mediators of Immune Regulation and Immunotherapy, Ed. Singhal SK. Elsevier Science Publishing Co., Inc., London, Canada, p. 213, 1985.

13) **Klinman DM** and Steinberg, AD: Autoimmunity, In: Systemic Lupus Erythematosus Textbook. Ed: Lahita RG. John Wiley Co., NY p. 1 1986.

14) **Klinman DM**, Lefkowitz MD, Barrett R, Honda M and Steinberg AD: Suppression of autoantibody production with anti-class II antibodies. In: Immune Intervention, Vol 2 Eds. Brochier J, Clot J and Sany J. Academic Press, NY. p. 87, 1986.

15) **Klinman DM**, Lefkowitz MD, Raveche ES, Honda M and Steinberg AD: Effect of anti-Ia treatment on the production of anti-DNA antibody by NZB mice. Eur. J. Immunol. 16:939, 1986.

16) **Klinman DM** and Steinberg AD: Idiotypy and autoimmunity. Arthritis Rheum. 29:697, 1986.

17) **Klinman DM**, and Steinberg AD: Proliferation of anti-DNA producing NZB B cell in a non-autoimmune environment. J. Immunol. 137:69, 1986.

18) **Klinman DM**, Mushinski JF, Honda M, Ishigatsubo Y, Mountz JD, Raveche ES and

Steinberg AD: Oncogene expression in autoimmune and normal peripheral blood mononuclear cells. J Exp. Med. 163:1292, 1986.

19) **Klinman DM**, Steinberg AD and Mushinski JF: Oncogene expression in angioimmunoblastic lymphadenopathy: Effect of cyclophosphamide therapy. Lancet 2:1055, 1986.

20) Steinberg AD, **Klinman DM**, Kastner DL, Seldin MF, Gause WC, Scribner CL, Britten JL, Siegel JM and Mountz JD: Genetic and molecular genetic studies of murine and human lupus. Proceedings of the First International Symposium on SLE. J. Rheumatol. (Sup) 13, 14:166, 1987.

21) **Klinman DM** and Cohen DI: Preserving primary cDNA libraries: Analyt. Biochem. 161:85, 1987.

22) Scribner CL, Hansen CT, **Klinman DM** and Steinberg AD: The interaction of the xid and me genes. J. Immunol. 138:3611, 1987.

23) **Klinman DM** and Steinberg AD: Systemic autoimmune diseases arise from polyclonal B cell activation. J. Exp. Med. 165:1755, 1987.

24) Mock B, Skurla R, Huppi K, D'Hoostelaere L, **Klinman DM** and Mushinski JF: A restriction fragment length polymorphism at the murine c-myb locus. Nuc. Acids Res. 15:4700, 1987.

25) **Klinman DM** and Steinberg AD: Preferential in vivo expansion of auto- antibody secreting B cells derived from normal DBA/2 mice. J. Immunol. 139:2284, 1987.

26) Ishigatsubo Y, **Klinman DM** and Steinberg AD: Transfer of anti-DNA producing B cells from NZB to unmanipulated xid recipients. Clin. Immunol. and Immunopathol. 45:244, 1987.

27) **Klinman DM** and Steinberg AD: Novel ELISA and ELISA spot assays used to quantitate B cells and serum antibodies specific for T cell and bromelated mouse red blood cell autoantigens. J. Immunol. Meth. 102:157, 1987.

28) Siegel J, Turner A, **Klinman DM**, Wilkinson M, Steinberg AD, MacLeod C, Paul WE, Davis M and Cohen DI: Sequence analysis of an X-linked lymphocyte-regulated gene family (XLR): homologies with the nuclear structural proteins, Lamins A and C. J. Exp. Med. 166:1702, 1987.

29) Steinberg AD, Seldin MF, Jaffe ES, Smith HR, **Klinman DM**, Krieg AM, and Cossman J. Angioimmunoblastic Lymphadenopathy with Dysproteinemia. Annals Int. Med. 108:575, 1988.

30) Steinberg AD and **Klinman DM**: Pathogenesis of Systemic Lupus Erythematosus. In: Rheumatic Disease Clinics of North America. Ed: Klippel JH. W.B. Saunders, Co. Phila, Pa. pp. 25-42. 1988.

31) Jensen L, Kuff EL, Wilson SH, Steinberg AD and **Klinman DM**: Antibodies from patients and mice with autoimmune disease react with recombinant HNRNP core protein A1. J. Autoimmunity 1:73, 1988.

32) **Klinman DM**, Ishigatsubo Y and Steinberg AD: Acquisition and maturation of expressed B cell repertoires in normal and autoimmune mice. J. Immunol. 141:801, 1988.

33) **Klinman DM**, Ishigatsubo Y and Steinberg AD: Pathogenesis of generalized autoimmunity: In vivo studies of the interaction between B cells and their internal milieu. Cell. Immunol. 17:360, 1988.

34) **Klinman DM**: B cells and autoimmune disease. ISI Atlas of Science 1:220, 1988.

35) **Klinman DM**, Blessing M, Hartman A and Steinberg AD: Natural autoantibodies and conventional antibodies show similar degrees of antigenic cross-reactivity. J. Clin. Invest. 82:652, 1988.

36) Ishigatsubo Y, Steinberg AD and **Klinman DM**: Genes which induce systemic autoimmunity also induce polyclonal B cell activation. Eur. J. Immunol. 18:1089, 1988.

37) Steinberg AD, **Klinman DM**, Krieg AM, Seldin MF and Kastner DL: Approach to the use of antigen non-specific immunosuppression in systemic lupus

erythematosus and other rheumatic autoimmune diseases. J Autoimmunity. 1:575, 1988.
38) **Klinman DM** and Steinberg AD: Systemic Lupus Erythematosus and Overlap Syndromes, In: Immunological Diseases, Ed. Samter M. Little Brown and Co. Boston, MA pp. 1335-1363, 1988.
39) Steinberg, AD, Krieg AM, Gause WC, Kastner DI, Mountz JD and **Klinman DM**: Cellular basis of immune modulation. In: Proceedings of the Nineteenth International Leukocyte Culture Conference, Ed. Kaplin JG and Green Dr. Alan R Liss Co., New York, NY pp 423-442, 1989.
40) **Klinman DM** and Morse HC: Characteristics of B cell proliferation and activation in murine AIDS. J. Immunol. 142:1144, 1989.
41) Steinberg AD, **Klinman DM**, Krieg AM, Seldin MF and Kastner DL: Approach to the use of antigen non-specific immunosuppression in systemic lupus erythematosus and other rheumatic autoimmune diseases. In: Immune Intervention in Autoimmune diseases Ed. Bach JF, Academic Press, San Diego. pp 79-96. 1989.
42) Huppi K, Bhatia K, Siwarski D, **Klinman DM**, Cherney B, and Smulson M: Sequence and organization of the mouse poly (ADP-ribose) polymerase gene._ Nuc Acids Res 17:3387, 1989.
43) **Klinman DM**: Regulation of B cell activation in autoimmune mice. Clin. Immunol. and Immunopath. 53:S25-34, 1989.

44) **Klinman DM**: Recent Advances in Systemic Lupus Erythematosus. Current Opinion in Immunology 1:740, 1989.
45) Steinberg AD, Scott DE, Gourley MF, and **Klinman DM**: Autoimmunity. In: Proceedings of the Fourth International Inflammation Research Meeting, 1989.
46) Steinberg AD, **Klinman DM** and Scott DE: From animal models to human pathology: facts and fantasies. In: Proceedings of the International League Against Rheumatism XVII. Ed. Chahade WH, Giorgi RD, Hirose-Pastor EM and Sato EI. Companhia Melhoramentos, Sao Paulo, Brazil. pp 2 - 5, 1989.
47) Steinberg AD, **Klinman DM** McIntyre TM and Kastner DL: Cellular and Molecular Basis of SLE. I. Genesis of autoantibodies. Proceedings of the Second International Congress of SLE. Ed. Boey ML, International Publishers, Singapore, pp 15 - 18, 1989.
48) **Klinman DM**, Eisenberg RA and Steinberg AD: Development of the autoimmune B cell repertoire in MRL-lpr/lpr mice. J. Immunol. 144:506, 1990.
49) Marsh JW and **Klinman DM**: Development of a diphtheria toxin mutant conjugate directed against antigen-specific B cells expressing high affinity surface Ig. J. Immunol. 144:1046, 1990.
50) Ishigatsubo Y, Sakamoto H, Hagiwara E, Aoki A, Tani K, Okubo T and **Klinman DM**: Quantitation of autoantibody-secreting B cells in systemic lupus erythematosus. Autoimmunity 5:71, 1990.
50) **Klinman DM**: IgG1 and IgG2a production by autoimmune B cells treated in vitro with Interleukin-4 and Interferon Gamma. J. Immunol. 144:2529, 1990.
52) D'Hoostelaere, LA and **Klinman DM**: Characterization of new mouse V kappa groups. J. Immunol. 145:2706, 1990.
53) **Klinman DM** and Holmes KL: The repertoire expressed by B lymphocytes *in vivo* varies in relation to cell phenotype and location. J. Immunol. 144:4520, 1990.
54) Huppi K, Siwarski D, Skurla R, **Klinman DM** and Mushinski JF: Pvt-1 transcripts are found in normal tissues and are altered by reciprocal (6;15) translocations in mouse plasmacytomas. Proc. Natl. Acad. Sci. (USA). 87:6964, 1990.
55) **Klinman DM**: Polyclonal B cell activation in (NZB X NZW)F1 X NZB backcross mice precedes and predicts the development of autoimmune-mediated glomerulonephritis and premature death. J. Clin. Invest 86:1249, 1990.

56) Steinberg AD, Krieg AM, Gourley MF and **Klinman DM**: Theoretical and experimental approaches to generalized autoimmunity. Immunol. Rev. 118:129, 1990.
57) **Klinman DM**, Higgins KW and Conover J: Sequential immunizations with rgp120's from independent isolates of HIV-1 induce the preferential expansion of broadly cross-reactive B cells. J. Exp. Med. 173:881, 1991.
58) Steinberg AD, Gourley MF, **Klinman DM**, Tsokos GC, Scott DE, and Krieg AM. Pathogenesis of systemic lupus erythematosus. Annals Internal Med 115:548, 1991.
59) **Klinman DM**, Krieg A, Conover J, Ussery MA and Black PL. Effect of cyclophosphamide, total body irradiation and zidovudine on retrovirus proliferation and disease progression in murine AIDS. Aids Research and Human Retroviruses 8:101, 1992
60) **Klinman DM**, Shirai A, Ishigatsubo Y, Conover J, and Steinberg AD. Quantitation of IgG and IgM secreting B cells in the peripheral blood of patients with systemic lupus erythematosus. Arth Rheum 34:1404, 1991.
61) **Klinman DM**. Similarities in B cell repertoire development between autoimmune and aging normal mice. J Immunol 148:1353, 1992.
62) Shirai A, Leitman-Klinman SF, and **Klinman DM**: HIV Infection induces both polyclonal and virus-specific B cell activation. J Clin Invest 89:561, 1992.
63) Krieg AM, Gourley MF, **Klinman DM**, Perl A, and Steinberg AD. Heterogeneous Expression and Induction of Endogenous Retroviral Sequences in Human Peripheral Blood Mononuclear Cells. AIDS Res Human Retroviruses 8:1991, 1992.
64) Shirai A and **Klinman DM**: Tolerance abnormalities in systemic lupus erythematosus. In: Dubois's Lupus Erythematosus, 4th Edition. Ed: Hahn, BH and Wallace, DJ. Lea and Febiger, Phila PA. pp 71 - 76, 1992.
65) **Klinman DM**. B cell abnormalities characteristic of systemic lupus erythematosus. In: Dubois's Lupus Erythematosus, 5th Edition. Ed: Hahn, BH and Wallace, DJ. Lea and Febiger, Phila PA. pp 195 - 206, 1997.
66) **Klinman DM**. Analysis of B lymphocyte cross-reactivity at the single cell level. J Immunol Meth 152:217, 1992.
67) **Klinman DM**, Steimer K, Glass W, and Conover J. Comparative immunogenicity of gp120-derived proteins and their induction of anti-V3 loop region antibodies. Journal of AIDS 5:1005, 1992.
68) **Klinman DM**: Introduction to the issues: viral vectors and potential problems in their use. Dev Biol Stand 76:299, 1992.
69) **Klinman DM**: Cross-reactivity of IgM secreting B cells from normal BALB/c mice. J Immunol 149:3569, 1992.
70) Shirai A, Holmes K and **Klinman DM**. Detection and quantitation of cells secreting IL-6 under physiologic conditions in BALB/c mice. J Immunol 150:1, 1992.
71) Leff RL and **Klinman DM**. PCR-dependent identification of Ig genes encoding autoantibodies. Immunomethods 1:169, 1992.
72) Wilde DB, **Klinman DM** and Bauer SR. Cytokine MAPPing in autoimmunity. Immunomethods 1:177, 1992.
73) **Klinman DM**. Autoimmunity; Concept and Experimental Models. Immunomethods 1:147, 1992.
74) **Klinman DM**, Dellacqua DK, Conover J and Huppi K. Vh gene utilization by IgG anti-DNA secreting lymphocytes derived from autoimmune MRL-lpr/lpr mice. Arthr Rheum 36:561, 1993.
75) McIntyre TM, **Klinman DM**, Rothman P, Lugo M, Basch JR, Mond JJ and Snapper CM. Transforming growth factor beta-1 selectively stimulated IgG2b secretion by LPS activated murine B cells. J Exp Med 177:1031, 1993.
76) **Klinman DM**. Cross reactivity of IgG and IgM secreting B cells in normal and autoimmune mice. J Autoimmunity 7:1, 1994.

77) Ishigatsubo Y, Igarashi T, Ohno S, Ueda A, Takao O and **Klinman DM**. Cross-reactivity of IgM and IgG secreting B cells in autoimmune mice. Arthr Rheum 36:1003, 1993.

78) **Klinman DM**, Holmes KL, Conover J, Chiang B and Gershwin ME. B-1 and conventional B cells from autoimmune NZB.H-2 bm12 mice exhibit similar functional characteristics in vivo. Eur J Immunol 23:1866, 1993.

79) Shirai A and **Klinman DM**. Immunization with recombinant gp160 prolongs the survival of HIV-1 transgenic mice. AIDS Res and Human Retroviruses. 9:979, 1993.

80) Mojcik CF, Gourley MF, **Klinman DM**, Krieg AM and Steinberg AD. Administration of a phosphorothioate oligonucleotide antisense to murine endogenous retroviral MCF env causes immune effects in vivo in a sequence-specific manner. Clin Immunol Immunopathol. 67:140, 1993.

81) Scribner C, Durfor C, **Klinman DM**, and Kozak R. Bioengineered Agents In: Scientific American's Introduction to Molecular Medicine Eds: Leder P, Clayton D and Rubenstein E. Scientific American Medicine Press, New York, NY, 1994.

82) **Klinman DM**. Natural antibodies, immune networks, and autoimmunity. In: Handbook of B and T cells. Ed: Snow EC. Academic Press, Inc, New York, NY. pp. 473 - 490, 1994.

83) **Klinman DM**, Shirai A, Conover J and Steinberg AD. Cross-Reactivity of IgG anti-DNA secreting B cells in patients with systemic lupus erythematosus. Eur J Immunol 24:53, 1994.

84) Shirai A, Sierra V, Kelly CI and **Klinman DM**. Individual cells simultaneously produce both IL-4 and IL-6 under physiologic conditions in vivo. Cytokine 6:329, 1994.

85) Schwartz DH, Cosentino LM, Shirai A, Conover J, Daniel S and **Klinman DM**: Lack of correlation between the number of circulating B cells and the concentration of serum antibodies reactive with the HIV-1 envelope glycoprotein. J Acquir Immune Defic Syndr 7:447, 1994.

86) **Klinman DM** and Nutman TB. ELISPOT assay to detect cytokine-secreting murine and human cells. In: Current Protocols in Immunology. Ed: Coligan JE and Strober W. Greene Publishing Associates, Brooklyn NY. Unit 6.19, 1994.

87) **Klinman DM**, Shirai A and Ishigatsubo Y: Polyclonal B cell activation and B cell cross-reactivity during autoantibody production in Systemic Lupus Erythematosus. Adv Exp Med Biol 115:124, 1994.

88) Shirai A, Aoki I, Otani M, Mond JH and **Klinman DM**: Treatment with dextran-conjugated anti-IgD delays the development of autoimmunity in MRL-lpr/lpr mice. J Immunol 153:1889, 1994.

89) Cosentino ML, Leitman SF and **Klinman DM**: HIV-specific B cells localize to the bone marrow of gp160-immunized mice and humans. Vaccine Research 3:195, 1994.

90) Schwieterman WD, Manasakis M, **Klinman DM** and Steinberg AD: Studies of marrow progenitor abnormalities in lupus-prone mice. Further studies of NZB Thy 1 neg Lin neg bone marrow cells. Clin Immunol and Immunopathol 72:114, 1994.

91) Shirai A, and **Klinman DM**. The genetic basis of autoimmune disease in MRL/lpr mice. Intern Rev Immunol 11:179, 1994.

92) Igarashi T, Ishigatsubo Y, Ohno S, Shirai A, **Klinman DM**: Cross-reactivity of IgM and IgG secreting B cells in murine chronic graft-versus-host reaction. Clin Immunol and Immunopathol 72:307, 1994.

93) **Klinman DM**, Haynes BF and Conover J: Activation of IL-4 and IL-6 secreting cells by HIV-specific synthetic peptides. AIDS Res and Human Retroviruses 11:97, 1995.

94) **Klinman DM** and Steinberg AD: Inquiry into murine and human lupus. Immunological Reviews 144:157, 1995.

95) Mor G, **Klinman DM**, Shapiro S, Hagiwara E, Sedegah M, Norman JA, Hoffman SL and Steinberg AD: Complexity of the cytokine and antibody response elicited by immunizing mice with PyCSP plasmid DNA. J Immunol 155:2039, 1995.

96) Aoki I, Kinzer C, Shirai A, Paul WE and **Klinman DM**: FcR^+ Non-B/Non-T

cells dominate the production of IL-4 and IL-6 in immunized mice. Proc Natl Acad Sci 92:2534, 1995.

97) Krieg AM, Yi A, Matson S, Waldschmidt TJ, Bishop G, Teasdale R, Koretzky GA and **Klinman DM**: CpG motifs in bacterial DNA trigger direct B cell activation. Nature 374:546, 1995.

98) Reeves JP, Lo C, **Klinman DM** and Epstein SL: Mouse mAbs to HIV gp120 generated by repeated immunization with gp120 from a single isolate, or by sequential immunization with gp120 from three isolates. Hybridoma 14:235, 1995.

99) Krause PR and **Klinman DM**: Efficacy, Immunogenicity, Safety and Use of the Live Attenuated Chickenpox Vaccine. J Pediatrics 127:518, 1995.

100) Shirai A, Conover J and **Klinman DM**: Increased activation and altered ratio of IFN-gamma : IL-4 secreting cells in MRL/lpr mice. Autoimmunity 21:107,1995.

101) Aoki I, Aoki A and **Klinman DM**: Activation of IL-4 and IL-6 secreting cells by antigen. Cytokine 7: 799, 1995.

102) Ishigatsubo Y, Steinberg AD, Krieg AM and **Klinman DM**: Increased utilization of polyreactive B cells during periods of generalized immune activation. Autoimmunity 22:113, 1995.

103) Hagiwara E, Abbasi F, Mor G, Ishigatsubo Y and **Klinman DM**: Phenotype and frequency of cells secreting IL-2, IL-4, IL-6, IL-10, IFNg and TNFa in human peripheral blood. Cytokine 7:815, 1995.

104) Hagiwara E and **Klinman DM**: Abnormalities in cytokine production and responsiveness in autoimmune disease. In: Cytokine Regulation of Humoral Immunity. Ed: Clifford Snapper. John Wiley and Sons, Ltd, New York, NY. pp. 409-430, 1996.

105) Hagiwara E, Sacks T, Leitman-Klinman SF and **Klinman DM**: Effect of HIV infection on the frequency of cytokine-secreting cells in human peripheral blood. AIDS Res and Human Retroviruses 12:127, 1996.

106) Hagiwara E, Gourley MF, Lee S and **Klinman DM**: Disease severity in patients with systemic lupus erythematosus correlates with an increased ratio of IL-10 : IFNg secreting cells in the peripheral blood. Arthr Rheum 39:379, 1996.

107) **Klinman DM**, Yi A, Beaucage SL, Conover J and Krieg AM: CpG motifs expressed by bacterial DNA rapidly induce lymphocytes to secrete IL-6, IL-12 and IFNg. Proc Natl Acad Sci 93:2879, 1996.

108) Aoki A, Taki A, Satoh K, Tanaka S, Ishii N, Minami M and **Klinman DM**: IL-3 promotes Th2 cell expansion through stimulating IL-4 production by splenic Non-T/B cells. Eur J Immunol 26:1388, 1996.

109) Chen S, Takeoka Y, Ansari A, Boyd R, **Klinman DM** and Gershwin ME: The natural history of disease expression in CD4 and CD8 gene deleted New Zealand black (NZB) mice. J Immunol 157:2676, 1996.

110) Yi, A, **Klinman DM**, Martin TL, Matson S and Krieg AM: Rapid immune activation by CpG motifs in bacterial DNA: Systemic induction of IL-6 transcription through an anti-oxidant sensitive pathway. J Immunol 157:5394, 1996.

111) Mor G, Yamshchikov G, Sedegah M, Takeno M, Wang R, Houghten RA, Hoffman S and **Klinman DM**: Induction of neonatal tolerance by a plasmid DNA vaccine. J Clin Invest 98:2700, 1996.

112) Hagiwara E, Adams EM, Plotz PH and **Klinman DM**: Abnormal cytokine production by peripheral blood mononuclear cells from patients with polymyositis and dermatomyositis. Clin Exp Rheum 14:485, 1996.

113) Snapper CM, Moorman MA, Rosas F, Shanebeck K, **Klinman DM**, Kehry MR, Mond JJ and Maliszewski R: IFNg is a potent inducer of Ig secretion by sort-purified murine B cells activated through the mIg, but not the CD40, signaling pathway. Int Immunol 8:877, 1996.

114) Young HA, **Klinman DM**, Reynolds DA, Grzegorzewski KJ, Mi A, Ward, JM, Winkler RT, Ortaldo JR, Kenny JJ and Koschlies KL: Bone marrow expression of IFNg results in severe B-cell lineage reduction, T-cell lineage alterations and hematopoietic progenitor

deficiencies. Blood 89:583, 1997.

115) Mor G, Singla M, Steinberg AD, Okuda K and **Klinman DM**: Do DNA vaccines induce autoimmune disease? Human Gene Therapy 8:293, 1997.

116) Sacks T and **Klinman DM**: Long-term effect of primary immunization on subsequent immune responsiveness. Cellular Immunology 177:162, 1997.

117) **Klinman DM**, Ichino M, Ichiro T, Gu M, Yamshchikov G and Conover J: Safety and Immunogenicity of DNA vaccines. Springer Sem Immunopath. 19:245, 1997.

118) **Klinman DM,** Yamshchikov G and Ishigatsubo, Y: Critical contribution of CpG motifs to the immunogenicity of plasmid DNA vaccines. J Immunol 158:3635, 1997.

119) Segal BM, **Klinman DM** and Shevach EM. Microbial products induce auto-immune disease by an IL-12 dependent process. J Immunol 158: 5087, 1997.

120) Hoffman SL, Doolan DL, Sedegah M, Wang R, Scheller LF, Kumar A, Weiss WR, Le TP, **Klinman DM,** Hobart P, Norman JA and Hedstrom RC: Toward clinical trials of DNA vaccines against malaria. Immunol and Cell Bio. 75:376, 1997.

121) Hagiwara E, Ishigatsubo Y and **Klinman DM**: B cell activation in patients with rheumatic disease: Association with an increased ratio of Th2: Th1 cytokine secreting cells. Jap J Rheum. 7:1, 1997.

122) Giese NA, Gabriele L, Doherty TM, **Klinman DM**, Tadesse-Heath L, Contursi C, Epstein SL and Morse HC: Interferon consensus sequence-binding protein, a transcription factor of the IFN regulatory factor family, regulates immune responses through control of IL-12 expression. J Exp Med 186:1535, 1997.

123) Karupiah G, Sacks TE, **Klinman DM**, Fredrickson TN, Hartley JW and Morse HC: Murine cytomegalovirus infection induces polyclonal B cell activation that is independent of CD4 T cells. Virology 240:12, 1998.

124) **Klinman DM**, Sechler JMG, Conover J, Gu M and Rosenberg AS: Contribution of cells at the site of DNA vaccination to the generation of antigen specific immunity and memory. J Immunol 160:2388, 1998.

125) Kanik KS, Hagiwara E, Schumacher R, Wilder RL and **Klinman DM**: Distinct patterns of cytokine secretion characterize new onset synovitis versus chronic rheumatoid arthritis. J Rheumatol 25:16, 1998.

126) Hagiwara E, Pando J, Ishigatsubo Y and **Klinman DM**: Altered frequency of type 1 cytokine secreting cells in the peripheral blood of patients with primary Sjogren's syndrome. J Rheumatol 25:89, 1998.

127) Verthelyi D, Dybdal N, Elias KA and **Klinman DM**: DNAse treatment delays anti-DNA autoantibody production but does not improve survival of lupus prone (NZB x NZW)F_1 mice. Lupus 7:233, 1998.

128) Gilkeson GS, Conover J, Halpern M, Pisetsky DS, Feagin A and **Klinman DM**: Effect of bacterial DNA on cytokine production by (NZB/NZW)F_1 mice J Immunol. 161:3890, 1998.

129) **Klinman DM**, Conover J, Bloom ET and Weiss W: Immunogenicity and efficacy of DNA vaccination in aged mice. Journal of Gerontology: Bio Sci 53A:B281, 1998.

130) Weiss WR, Ishii KJ, Hedstrom RC, Sedegah M, Barnhart K, **Klinman D** and Hoffman SL: A plasmid encoding murine GM-CSF increases protection conferred by a malaria DNA vaccine. J Immunol. 161:2325, 1998.

131) **Klinman DM:** Therapeutic applications for CpG-containing oligodeoxy-nucleotides. Antisense Nucl Acid Drug Dev. 8:181, 1998.

132) Song X, Gu M, Jin W, **Klinman DM** and Wahl, SM: Plasmid DNA encoding TGF-β1 suppresses chronic disease in a streptococcal cell wall-induced arthritis model. J Clin Invest 101:2615, 1998.

133) Wang R, Doolan DL, Charoenvit Y, Hedstrom RC, Gardner MJ, Hobart P, Tine J, Sedegah M, Fallarme V, Sacci JB, Kaur M, **Klinman DM**, Hoffman SL and Weiss WR: Simultaneous induction of multiple antigen-specific CTL in non-human primates by immunization

with a mixture of 4 Plasmodium falciparum DNA plasmids. Infect Immun. 66:4193, 1998.
134) Gu M, Leppla SH, and **Klinman DM**: Protection against Anthrax toxin by vaccination with a DNA plasmid encoding anthrax protective antigen. Vaccine 17:340, 1999.
135) Elkins KL, Rhinehart-Jones TR, Stibitz S, Conover JS and **Klinman DM**: Bacterial DNA protects mice against lethal infection by intracellular bacteria. J Immunol. 162:2291, 1999.
136) **Klinman DM**, Barnhart KM and Conover J. CpG motifs as immune adjuvants. Vaccine 17:1,1999.
137) Verthelyi D and **Klinman DM**: Impact of the type 1 : type 2 cytokine imbalance on disease activity in SLE. In: Lupus- Cellular and Molecular Pathogenesis. Eds: Kammer GM and Tsokos GC, Humana Press, Totowa NJ. 1999.
138) Ishii KJ, Weiss WR, Ichino M, Verthelyi D, Hoffman SL and **Klinman DM**: Activity and safety of DNA plasmids encoding IL-4 and IFNg. Gene Ther. 6:237,1999.
139) **Klinman DM**, Conover J, Leiden JM, Rosenberg AS and Sechler JMG: Safe and effective regulation of hematocrit by gene gun administration of an erythropoietin-encoding DNA plasmid. Hum Gene Ther 10:659, 1999.
140) Suziki K, Mori A, Ishii KJ, Saito J, Singer DS, **Klinman DM**, Krause PR and Kohn LD: Activation of target tissue immune recognition molecules by double strand polynucleotides. PNAS 96:2285, 1999.
141) Ichino M, Mor G, Conover J, Weiss W, Takeno M, Ishii KJ and **Klinman DM**: Factors associated with the development of neonatal tolerance after the administration of a plasmid DNA vaccine. J Immunol 162:3814, 1999.
142) Sur S, Wild JS, Alam R, Sur Nilanjana, **Klinman DM**: Long-term prevention of allergic lung inflammation in a mouse model of asthma by CpG oligodeoxynucleotides. J Immunol 162:6284, 1999.
143) Ishii, KJ, Weiss, WR and **Klinman, DM**: Prevention of neonatal tolerance by a plasmid encoding GM-CSF. Vaccine 18:703, 1999.
144) **Klinman DM**, Verthelyi D, Takeshita F and Ishii KJ: Immune recognition of foreign DNA: A cure for bioterrorism? Immunity 11:123, 1999.
145) Calarota SA, Leandersson A, Bratt G, Hinkula J, **Klinman DM**, Weinhold KJ and Wahren B: Immune responses in asymptomatic HIV-1 patients after HIV-DNA immunization followed by highly active anti-retroviral treatment. J. Immunol 163:2330, 1999.
146) Goeckeritz BE, Flora M, Witherspoon K, Vos Q, Lees A, Dennis GJ, Pisetsky DS, **Klinman DM**, Snapper CM and Mond JJ: Multivalent cross-linking of membrane Ig sensitizes murine B cells to a broader spectrum of CpG-containing oligodeoxynucleotide motifs, including their methylated counterparts, for stimulation of proliferation and Ig secretion. Int Immunol 11:1693, 1999.
147) Aoki I, Itoh S, Yokota S, Tanaka S, Ishii N, Okuda K, Minami M, and **Klinman DM**: Contribution of mast cells to the T helper 2 response induced by simultaneous subcutaneous and oral immunization. Immunology 98:519, 1999.
148) **Klinman DM**, Conover J and Coban C: Repeated Administration of CpG oligonucleotides provides long-term protection against bacterial infection. Infection and Immunity 67:5658, 1999.
149) **Klinman DM,** Ishii KJ and Verthelyi D: CpG DNA Augment the Immunogenicity of plasmid DNA Vaccines. Current Topics Micro Immunol. 247:131, 2000.
150) **Klinman DM**, Kamstrup S, Verthelyi D, Gursel I, Ishii KJ, Takeshita F and Gursel M: Activation of the Innate Immune System by CpG Oligodeoxynucleotides: Immunoprotective Activity and Safety Issues. Springer Sem Immunopath. 22:173, 2000.
151) Takeshita F, Ishii KJ, Ueda A, Ishigatsubo Y and **Klinman DM**: Positive and negative regulatory elements contribute to CpG ODN mediated regulation of human IL-6 gene expression. Eur J Immunol 30:108, 2000.

152) Kusakabe K, Xin K, Katoh H, Sumino K, Hagiwara E, Kawamoto S, Okuda K, Miyagi Y, Aoki I, Nishioka K, **Klinman DM** and Okuda K: The timing of GM-CSF expression plasmid administration influences the Th1/Th2 response induced by an HIV-1 specific DNA vaccine. J Immunol 164:3102, 2000.

153) **Klinman DM**, Takeshita K, Kamstrup S, Takeshita S, Ishii K, Ichino M and Yamada H: DNA Vaccines: Capacity to induce autoimmunity and tolerance. Developments and Clinical Progress of DNA Vaccines. Dev Biol Stand. Basel, Karger, 104:45, 2000.

154) Krause PR and **Klinman DM**: Varicella Vaccination: Evidence for frequent reactivation of the vaccine strain in healthy children. Nature Med 6:451, 2000.

155) Gurunathan S, **Klinman DM** and Seder RA: DNA Vaccines: Immunology, application and optimization. Ann Rev Immunol. 18:927, 2000.

156) Verthelyi D and **Klinman DM**: Sex hormone levels correlate with the activity of cytokine-secreting cells in vivo. Immunology 100:384, 2000.

157) Freidag BL, Melton GB, Collins F, **Klinman DM**, Cheever A, Stobie L, Suen W and Seder RA: CpG oligodeoxynucleotides and interleukin-12 improve the efficacy of mycobacterium bovis BCG vaccination in mice challenged with M. tuberculosis. Infect Immun. 68:2948, 2000.

158) Takeshita F and **Klinman DM**: CpG ODN-mediated regulation of IL-12 p40 transcription. Eur J Immunol 30:1967, 2000.

159) Krause PR and **Klinman DM**: Varicella Vaccine Revisited. Nature Med 6:1300, 2000.

160) Verthelyi D, Ishii KJ, Gursel M and **Klinman DM**: Human peripheral blood cells differentially recognize and respond to two distinct CpG motifs. J Immunol 166:2372, 2001.

161) Stan AC, Casares S, Brumeanu, T, **Klinman DM** and Bona CA: CpG motifs of DNA vaccines induce the expression of chemokines and MHC class II molecules on myocytes. Eur J Immunol 31:301, 2001.

162) Kamstrup S, Verthelyi D, **Klinman DM**: Response of porcine peripheral blood mononuclear cells to CpG-containing oligodeoxynucleotides. Vet Microbiol. 78:353, 2001.

163) Takeshita S, Takeshita F, Haddad DE, Ishii K and **Klinman DM**: CpG oligodeoxynucleotides induce murine macrophages to up-regulate expression of chemokine mRNA. Cell Immunol 15:101, 2000.

164) Smith HA and **Klinman DM**: The Regulation of DNA Vaccines. Curr Opin in Biotech. 12:299, 2001.

165) **Klinman DM** and Krause PR: Disseminated rashes and varicella vaccine.Pediatr Infect Dis J. 20:467, 2001.

166) Verthelyi D, Petri M, Ylamus M, and **Klinman DM**: Dissociation of sex hormone levels and cytokine production in SLE patients. Lupus 10:352, 2001.

167) Takeshita S, Takeshita F, Haddad DE, Janabi N and **Klinman DM**: Activation of microglia and astrocytes by CpG oligodeoxynucleotides. NeuroReport 12:3029, 2001.

168) Ishii KJ, Suzuki K, Coban C, Takeshita F, Itoh T, Matoba H, Kohn LD and **Klinman DM**: Genomic DNA released by dying cells induces the maturation of APCs. J Immunol 167:2602, 2001.

169) Takeshita F, Leifer CA, Gursel I, Ishii KJ, Takeshita S, Gursel M and **Klinman DM**: Cutting Edge: Role of toll-like receptor 9 in CpG DNA-induced activation of human cells. J

Immunol 167:3555, 2001.

170) Gursel I, Gursel M, Ishii KJ and **Klinman DM**: Sterically stabilized cationic liposomes improve the uptake and immunostimulatory activity of CpG oligodeoxynucleotides. J Immunol 167:3324, 2001.

171) Okuda K, Xin K, Haruki A, Kawamoto S, Kojima Y, Hirahara F, Okada H, **Klinman DM** and Hamajima K: Transplacental genetic immunization after intravenous delivery of plasmid DNA to pregnant mice. J Immunol 167: 5478, 2001.

172) Kumar S, Villinger F, Oakley M, Aguiar JC, Jones TR, Hedstrom RC, Gowda K, Chute J, Stowers A, Kaslow DC, Thomas EK, Tine J, **Klinman DM**, Hoffman SL, Weiss WW: A DNA vaccine encoding the 42 kDa C-terminus of merozoite surface protein 1 of Plasmodium falciparum induces antibody, interferon-gamma and cytotoxic T cell responses in rhesus monkeys: immuno-stimulatory effects of granulocyte macrophage-colony stimulating factor. Immunol Lett. 8:13, 2002

173) Verthelyi D, Kenney RT, Seder RA, Gam AA, Friedag B, **Klinman DM:** CpG oligodeoxynucleotides as vaccine adjuvants in primates. J Immunol. 168:1659, 2002.

174) Gursel M, Verthelyi D, Gursel I, Ishii KJ, **Klinman DM.** Differential and competitive activation of human immune cells by distinct classes of CpG oligodeoxynucleotide. J Leukoc Biol. 71:813, 2002.

175) **Klinman DM**, Ishii KJ, and Gursel I: Immunoprotective Activity of CpG Oligonucleotides. Microbial DNA and Immune Modulation. Humana Press. Totowa, NJ. Ed. Raz, E. p. 255, 2002.

176) **Klinman DM**, Takeshita F, Gursel I, Leifer C, Ishii KJ, Verthelyi, D, Gursel M. CpG DNA: Recognition by and activation of monocytes. Microbes and Infection 4:897, 2002.

177) Verthelyi D and **Klinman DM**: CpG Oligonucleotides: Safety Considerations. Microbial DNA and Immune Modulation. Humana Press. Totowa, NJ. Ed. Raz, E. p. 385, 2002.

178) Zheng M, **Klinman DM**, Gierynska M, Rouse BT: DNA containing CpG motifs induces angiogenesis. Proc Natl Acad Sci 99:8944, 2002.

179) Ishii KJ, Takeshita T, Gursel I, Gursel M, Conover J, Nussenzweig A and **Klinman DM**: Potential role of phosphatidylinositol 3 kinase, rather than DNA-dependent protein kinase, in CpG DNA-induced immune activation. J Exp Med 196:269, 2002.

180) Zeuner RA, Ishii KJ, Lizak MJ, Gursel I, Yamada H, **Klinman DM** and Verthelyi D: Suppressive Oligodeoxynucleotides reduce CpG-induced arthritis. Arthritis Rheum 46:2219, 2002.

181) Gursel M, Verthelyi D and **Klinman DM.** CpG oligonucleotides induce human monocytes to mature into functional dendritic cells. Eur J Immunol. 32:2617, 2002.

182) Rhee EG, Mendez S, Shah JA, Wu CY, Kirman JR, Turon TN, Davey DF, Havis H, **Klinman DM**, Coler RN, Sacks DL and Seder RA. Vaccination with heat-killed leishmania antigen or recombinant leishmanial protein and CpG oligodeoxynucleotides induces long-term memory CD4+ and CD8+ T cell responses and protection against leishmania major infection. J Exp Med. 195:1565, 2002.

183) Kojima Y, Xin KQ, Ooki T, Hamajima K, Oikawa T, Shinoda K, Ozaki T, Hoshino Y, Hounai N, Nakazawa M, **Klinman DM** and Okuda K. Adjuvant effect of multi-CpG motifs on an HIV-1 DNA vaccine. Vaccine 20:2857, 2002.

184) **Klinman DM**, Zheng M, Gierynska M and Rouse BT. DNA containing bioactive CpG motifs promotes angiogenesis. Drug News and Perspectives 15:358, 2002.

185) Yamada H, Gursel I, Takeshita F, Conover J, Ishii KJ, Gursel M, Takeshita S and **Klinman DM.** Effect of suppressive DNA on CpG-induced immune activation. J Immunol 169:5590, 2002.

186) Choudhury BK, Wild JS, Alam R, **Klinman DM**, Boldogh I, Dharajiya N, Mileski WJ and Sur S. In vivo role of p38 MAPK in mediating the anti-inflammatory effects of CpG ODN in murine asthma. J Immunol 169:5955, 2002.

187) Xin K-Q, Ooki T, Jounai N, Hashimoto H, Kojima K, Hirai k, **Klinman DM**, Ozawa K and

Okuda K. A DNA Vaccine Containing Inverted Terminal Repeats from Adeno-Associated Virus Increases Immunity to HIV. J Gene Medicine 5:438, 2003.

188) Xin K-Q, Ooki T, Mizukami h, Hamajima K, Okudela K, Hashimoto K, Kojima Y, Jounai N, Kumamoto Y, Sasaki S, K, **Klinman DM,** Ozawa K and Okuda K. Oral administration of recombinant adeno-associated virus elicits HIV-specific immune responses. Hum Gene Ther 13:1571, 2002.

189) **Klinman DM** and Smith HA. DNA Vaccines: Safety and Regulatory Issues. DNA Vaccines. Ed: HCJ Ertl. Kluwer Academic/Plenum Publishers. Georgetown, TX, 2003.

190) Zeuner RA, **Klinman DM**, Illei G, Yarboro Ch, Ishii K, Gursel M and Verthelyi D. Response of PBMC from lupus patients to stimulation by CpG oligodeoxynucleotides. Rheum. 42:573, 2003.

191) **Klinman DM**, Ishii K, Gursel M, Gursel I, Takeshita S and Takeshita F: Immunotherapeutic Applications of CpG-containing Oligodeoxynucleotides. Drug News and Perspectives 13:289, 2003.

192) Mazzoni A, Leifer CA., Mullen GED, Kennedy MN, **Klinman DM,** and Segal DM. Histamine inhibits IFNg release from plasmacytoid dendritic cells. J. Immunol. 170:2269, 2003.

193) Verthelyi D, Gursel M, Kenney KT, Lifson JD, Liu S, Mican J and **Klinman DM.** CpG oligodeoxynucleotides protect normal and SIV-infected macaques from Leishmania infection. J. Immunol. 170;4717, 2003.

194) Jounai N, Okuda K, Kojima Y, Toda Y, Hamajima K, Ohba K, **Klinman DM** and Xin K. Contribution of the rev gene to the immunogenicity of DNA vaccines targeting the envelope glycoprotein of HIV. J Gene Med 5:609, 2003.

195) Leifer C, Verthelyi D and **Klinman DM.** Human Response to Immunostimulatory CpG Oligodeoxynucleotides. J Immunother. 26:313, 2003.

196) **Klinman DM.** CpG DNA as a vaccine adjuvant. Expert Rev. Vaccines 2:305, 2003.

197) **Klinman DM** and Currie D. Hierarchical recognition of CpG motifs in immunostimulatory oligodeoxynucleotides. Clin Exp Immunol 133:227, 2003.

198) Prince GA, Mond JJ, Porter DD, Yim KC Lan SJ and **Klinman DM.** Immunoprotective activity and safety of a respiratory syncytial virus vaccine: mucosal delivery of fusion glycoprotein with a CpG oligodeoxynucleotide adjuvant. J. Virol. 77:13156, 2003.

199) **Klinman DM**, Zeuner R, Yamada H, Gursel M, Currie D and Gursel I. Regulation of CpG-Induced Immune Activation by Suppressive Oligodeoxynucleotides. Annals NY Acad Sci 1002; 112, 2003.

200) Xin K, Hoshino Y, Toda Y, Igigi S, Kojima Y, Jounai N, Ohba K, Cachoeira A, Kiwaki M, Hamajima K, **Klinman DM** and Okuda K. Immunogenicity and protective efficacy of orally administered recombinant *Lactococcus lactis* expressing surface-bound HIV Env. Blood 102:223, 2003.

201) Verthelyi D and **Klinman DM.** Immunoregulatory Activity of CpG Oligonucleotides in Humans and Non-Human Primates. Clin Immunol 109:64, 2003.

202) Zeuner RA, Verthelyi D, Gursel M, Ishii KJ and **Klinman DM.** Influence of stimulatory and suppressive DNA motifs on host susceptibility to inflammatory arthritis. Arthritis Rheum 48:1701, 2003.

203) **Klinman DM.** Does activation of the innate immune system contribute to the development of rheumatoid arthritis? Arthritis Rheum 48:590, 2003.

204) Mendez S, Tabbara K, Belkaid Y, Bertholet S, Verthelyi D, **Klinman DM**, Seder RA and Sacks DL. Co-injection of CpG-Containing Immunostimulatory Oligodeoxynucleotides Reduces the Pathogenicity of Live Vaccine against Cutaneous Leishmaniasis but Maintains Its Potency and Durability. Infect Immun 71:5121, 2003.

205) Gursel I, Yamada H, Gursel M, Coban C, Conover J and **Klinman DM**. Repetitive elements in mammalian telomeres suppress bacterial DNA induced immune activation. J Immunol 171:1393, 2003.

206) Ishii KJ, Kawakami K, Gursel I, Conover J, Joshi BH, **Klinman DM** and Puri RK. Antitumor therapy with bacterial DNA and toxin: complete regression of established tumor induced by liposomal CpG oligonucleotides plus IL-13 cytotoxin. Clin Cancer Res. 9:6516, 2003.

207) Coban D, Ishii KJ, Stowers AW, Keister DB, **Klinman DM** and Kumar N. Effect of CpG Oligodeoxynucleotides on the Immunogenicity of Pfs25, a Plasmodium falciparum transmission blocking vaccine antigen. Infect Immunity 72:584, 2004.

208) **Klinman DM.** Immunotherapeutic Uses of CpG Oligodeoxynucleotides. Nature Rev Immunology 4:249, 2004.

209) Takeshita F and **Klinman DM.** Signal transduction pathways mediated by the interaction of CpG DNA with Toll-like receptor 9. Seminars in Immunology. 16:17, 2004.

210) Isaguliants MG, Iakimtchouk K, Petrakova NV, Yermalovich MA, Zuber AK, Kashuba VI, Belikov SV, Andersson S, Kochetkov SN, **Klinman DM** and Wahren B. Gene immunization may induce secondary antibodies reacting with DNA. Vaccine 29:1576, 2004.

211) Verthelyi D, Wang VW, Lifson JD and **Klinman DM.** CpG oligodeoxynucleotides improve the response to hepatitis B immunization in healthy and SIV infected rhesus macaques. AIDS 20:1003, 2004

212) Ishii KJ, Gursel I, Gursel M and **Klinman DM.** Immunotherapeutic utility of stimulatory and suppressive oligodeoxynucleotides. Curr Op Mol Ther 6:166, 2004.

213) Dong L, Ito S, Ishii, KJ and **Klinman DM.** Suppressive oligodeoxy-nucleotides protect against the development of collagen-induced arthritis in mice. Arthr Rheum 50:1686, 2004.

214) **Klinman DM**, Ito S, Currie D and Ishii KJ. Use of CpG oligodeoxynucleotides as immunoprotective agents. Expert Opinion BioTech. 4:937, 2004.

215) Ito S, Ishii KJ, Shirota H and **Klinman DM**. CpG oligodeoxynucleotides improve the survival of pregnant and fetal mice following *Listeria monocytogenes* infection. Infect Immunity 72:3543, 2004.

216) Tsujimura H, Tamura T, Kong JG, Nishiyama A, Ishii KJ, **Klinman DM** and Ozato K. Toll-like receptor 9 signaling activates NF-kappaB through IFN regualtory factor-8/IFN consensus sequence binding protein in dendritic cells. J Immunol 172:6820, 2004.

217) Takeshita F, Suzuki K, Sasaki S, Ishii N, **Klinman DM**, and Ishii KJ. Transcriptional regulation of the human Toll-like receptor 9 gene. J Immunol. 173:2552, 2004.

218) **Klinman DM**, Xie H, Little SF, Currie D and Ivins B. CpG oligodeoxynucleotides improve the protective immune response induced by anthrax vaccination of rhesus macaques. Vaccine 22:2881, 2004.

219) **Klinman DM**, Currie D, Gursel I and Verthelyi, D. Use of CpG Oligodeoxynucleotides as Immune Adjuvants. Immunol Rev. 199:201, 2004.

220) Shinoda S, Xin KQ, Jounai N, Kojima Y, Tamura Y, Okada E, Kawamoto S, Okuda K, **Klinman DM** and Okuda K. Polygene DNA vaccine induces a high level of protective effect against HIV-vaccinia virus challenge in mice. Vaccine 22:3676, 2004.

221) Gurnathan S, **Klinman DM** and Seder RA. Immune enhancing sequences (CpG motifs), cytokines and other immunomodulatory moieties. In: New Generation Vaccines. Eds Levine MM, Kaper JB, Rappuoli R, Liu MA and Good MF. Marcel Dekker, Inc, New York, NY, 2004.

222) Shirota H, Gursel M and **Klinman DM.** Suppressive oligodeoxynucleotides inhibit Th1 differentiation by blocking IFNg and IL-12 mediated signaling. J Immunol. 173:5002, 2004.

223) Yamada H, Ishii KJ and **Klinman DM.** Suppressive oligodeoxynucleotides inhibit CpG-induced inflammation of the mouse lung. Crit Care Med 32:2045, 2004.

224) Sen G, Flora M, Chattopadhyay G, **Klinman DM**, Lees A, Mond JJ and Snapper CM. The critical DNA flanking sequences of a CpG oligodeoxynucleotide, but not the 6 base CpG motif, can be replaced with RNA without quantitative or qualitative changes in Toll-like receptor 9-mediated activity. Cell Immunol 232:64, 2004.

225) Dong L, Ito S, Ishii KJ and **Klinman DM**: Suppressive oligodeoxynucleotides delay the onset of glomerulonephritis and prolong survival in lupus-prone NZB x NZW mice. Arthr Rheum 52:651, 2005.

226) Sugiyama T, Gursel M, Takeshita F, Coban C, Conover J, Kaisho T, Akira S, **Klinman DM** and Ishii KJ: CpG RNA: Identification of Novel Single-Stranded RNA That Stimulates Human CD14+CD11c+ Monocytes. J Immunol. 174:2273, 2005.

227) Xie H, Gursel I, Ivins BE, Singh M, O'Hagan DT, Ulmer JB and **Klinman DM.** CpG Oligodeoxynucleotides Adsorbed onto PLG Microparticles Improve the Immunogenicity and Protective Activity of the Licensed Anthrax Vaccine. Infect Immunity 73:828, 2005.

228) Ito S, Ishii KJ, Gursel M, Ihata A. Shirota J and **Klinman DM:** CpG Oligodeoxynucleotides Enhance Neonatal Resistance to Listeria Infection. J Immunol. 174:777, 2005.

229) Shirota H, Gursel I, Gursel M and **Klinman DM.** Suppressive oligodeoxynucleotides protect mice from lethal endotoxic shock. J Immunol. 174:4579, 2005.

230) Ito S, Ishii KJ, Ihata A and **Klinman DM.** Contribution of nitric oxide to CpG-mediated protection against Listeria monocytogenese. Infect Immun. 73:3803, 2005.

231) Tamura Y, Hamajima K, Matsui K, Yanoma S, Naita M, Tajima N, Xin KQ, **Klinman DM** and Okuda K. The F(ab')2 fragment of an Abeta-specific monoclonal antibody reduces abeta deposits in the brain. Neurobiol Dis. 20:541, 2005.

232) Dalloul RA, Lillehoj HS, **Klinman DM**, Ding X, Min W, Heckert RA and Lillehoj EP. In ovo administration of CpG oligodeoxynucleotides and the recombinant microneme protein MIC2 protects against Eimeria infections. Vaccine 23:3108, 2005.

233) Coban D, Ishii KJ, Gursel M, **Klinman DM** and Kumar N. Effect of plasmid backbone modification by different human CpG motifs on the immunogenicity of DNA vaccine vectors. J Leukoc Biol. 78:647, 2005.

234) Xin KQ, Jounai N, Someya K, Honma K, Mizugushi H, Naganawa S, Kitamura K, Hayakawa T, Saha S, Takeshita F, Honda M, **Klinman DM** and Okuda K. Prime-boost vaccination with plasmid DNA and a chimeric adenovirus type 5 vector with type 35 fiber induces protective immunity against HIV. Gene Ther.12:1769, 2005.

235) Takeshita F, Ishii KJ, Kobiyama K, Kojima Y, Coban C, Sasaki S, Ishii N, **Klinman DM**, Okuda K, Akira S and Suzuki K. TRAF4 acts as a silencer in TLR-mediated signaling through the association with TRAF6 and TRIF. Eur J Immunol. 35:2477, 2005.

236) Ishii KJ, Ito S, Tamura T, Hemmi H, Conover J, Ozato K, Akira S and **Klinman DM.** CpG-activated Thy1.2+ dendritic cells protect against lethal Listeria monocytogenes infection. Eur J Immunol. 35:2397, 2005.

237) Lugo-Villarino G, Ito S, **Klinman DM** and Glimcher LH. The adjuvant activity of CpG DNA requires T-bet expression in dendritic cells. Proc Natl Acad Sci. 102:13248, 2005.

238) Ito S, Pedras-Vasconcelos J and **Klinman DM.** CpG oligonucleotides increase the susceptibility of normal mice to infection by Candida albicans. Infect Immun 73:6154, 2005.

239) **Klinman DM**, Gursel I, Klaschik S, Dong L, Currie D and Shirota H. Therapeutic potential of oligonucleotides expressing immunosuppressive TTAGGG motifs. Ann NY Acad Sci 1058:87, 2005.

240) Belyakov IM, Kuznetsov VA, Kelsall B, **Klinman DM**, Moniuszko M, Lemon M, Markham PD, Pal R, Clements JD, Lewis MG, Strober W, Franchini G and Berzofsky JA. Impact of vaccine-induced mucosal high avidity CD8+ CTL in delay of AIDS-viral dissemination from mucosa. Blood 107:3258, 2006.

241) Gursel M, Gursel I, Mostowski HS and **Klinman DM.** CXCL16 influences

the nature and specificity of CpG-induced immune activation. J Immunol 177:1575, 2006.

242) Shirota H, Ishii KJ, Takakuwa and **Klinman DM.** Contribution of interferon-beta to the immune activation induced by double-stranded DNA. Immunology 118:302, 2006.

243) **Klinman DM.** CpG oligonucleotides accelerate and boost the immune response elicited by AVA, the licensed anthrax vaccine. Expert Rev Vaccines. 5:365, 2006.

244) **Klinman DM.** Adjuvant activity of CpG oligonucleotides. Int Rev Immunol. 25:135, 2006.

245) Saha S, Takeshita F, Sasaki S, Matsuda T, Tanaka Tozuka M, Takase K, Matsumoto T, Okuda K, Ishii N, Yamaguchi K, **Klinman DM**, Xin KQ and Okuda K. Multivalent DNA vaccine protects mice against pulmonary infection caused by Pseudomonas aeruginosa. Vaccine. 11:6240, 2006.

246) Takeshita F, Tanaka T, Matsuda T, Tozuka M, Kobiyama K, Saha S, Matsui K, Ishii KJ, Coban C, Akira S, Ishii N, Suzuki K, **Klinman DM**, Okuda K and Sasaki S. Toll-like receptor adaptor molecules enhance DNA-raised adaptive immune responses against influenza and tumors through activation of innate immunity. J Virol 80:6218, 2006.

247) Belyakov IM, Isakov D, Zhu Q, Dzutsev A, **Klinman DM** and Berzofsky JA. Enhancement of CD8+ T cell immunity in the lung by CpG ODN increases protective efficacy of a Modified Vaccinia Ankara vaccine against lethal poxvirus infection even in CD4-deficient hosts. J. Immunol. 177:6336, 2006.

248) Xin KQ, Mizukami H, Urabe M, Toda Y, Shinoda K, Yoshida A, Oomura K, Kojima Y, Ichino M, **Klinman DM**, Ozawa K and Okuda K. Induction of Robust Immune Responses against HIV is Supported by the Inherent Tropism of AAV5 for DC. J Virol 80:11899, 2006.

249) Fujimoto C, Yu C, Shi G, Vistica BP, Wawrousek EF, **Klinman DM**, Chan C, Egwuagu EC and Gery I. Pertussis toxin is superior to TLR ligands in enhancing pathogenic autoimmunity, targeted at a neo-self antigen, by triggering robust expansion of Th1 cells and their cytokine production. J Immunol 177:6896, 2006.

250) Saha S, Yoshida S, Ohba K, Matsui K, Matsuda T, Takeshita F, Umeda K, Tamura Y, Okuda K, **Klinman D**, Xin KQ and Okuda K. A fused gene of nucleoprotein (NP) and herpes simplex virus genes (VP22) induces highly protective immunity against different subtypes of influenza virus. Virology 354:48, 2006.

251) **Klinman DM**, Xie H and Ivins BE. CpG oligonucleotides improve the protective immune response induced by the licensed anthrax vaccine. Annals NY Acad Sci 1082:137, 2006.

252) Fogg CN, America JL, Lustig S, Huggins JW, Smith SC, Damon I, Resch W, Earl PL, **Klinman DM** and Moss B. Adjuvant-enhanced antibody responses to recombinant proteins correlates with protection of mice and monkeys to orthopoxvirus challenges. Vaccine 25:2787, 2007.

253) Klaschik S, Gursel I and **Klinman DM.** CpG-mediated changes in gene expression in murine spleen cells identified by microarray analysis. Molec Immunol. 44:1095, 2007.

254) Saha S, Takeshita F, Matsuda T, Jounai N, Kobiyama K, Matsumoto T, Sasaki S, Yoshida A, Xin KQ, **Klinman DM**, Uematsu S, Ishii KJ, Akira S and Okuda K. Blocking of the TLR5 Activation Domain Hampers Protective Potential of Flagellin DNA Vaccine. J Immunol. 179:1147, 2007.

255) Shirota H, Petrenko L, Hong C and **Klinman DM.** Potential of transfected muscle cells to contribute to DNA vaccine immunogenicity. J. Immunol. 179:329, 2007.

256) **Klinman DM**, Currie D, Lee G, Grippe V and Merkel T. Systemic but not mucosal immunity induced by AVA prevents inhalational anthrax. Microbes Infect. 9:1478, 2007.

257) Yasuda H, Leelahavanichkul A, Tsunoda S, Dear JW, Takahashi Y, Ito S, Hu X, Zhou H, Doi K, Childs RW, **Klinman DM,** Yuen PS and Star RA. Chloroquine and inhibition of Toll-like receptor 9 protect from sepsis-induced acute kidney injury. Am J Physiol Renal Physiol. 294:F1050, 2008.

258) **Klinman DM**, Currie D, Fujimoto C, Gery I and Shirota H. Therapeutic potential of immunosuppressive oligonucleotides expressing TTAGGG motifs. In: Nucleic Acids in Innate Immunity, Eds: Ishii KJ and Akira S. CRC Press, New York, NY. 2008.

259) Sato T, Shimosato T, Alvord WG and **Klinman DM.** Suppressive oligodeoxynucleotides inhibit silica-induced pulmonary inflammation. J. Immunol. 180:7648, 2008.

260) **Klinman DM**, Shirota H, Tross D, Sato T and Klaschik S. Synthetic oligonucleotides as modulators of inflammation. J Leukoc Biol. 84:958, 2008.

261) **Klinman DM.** ELISPOT assay to detect cytokine-secreting murine and human cells. In: Current Protocols in Immunology. Section 6.19, p.1-9. (In Press).

262) Shimada M, Wang HB, Kondo A, Xu XP, Yoshida A, Shinoda K, Ura T, Mizuguchi H, **Klinman D**, Luo JL, Bai H, Okuda K. Effect of therapeutic immunization using Ad5/35 and MVA vectors on SIV infection of rhesus monkeys undergoing antiretroviral therapy. Gene Therapy (In Press).

263) Zhu Q, Egelston C, Vivekanandhan A, Uematsu S, Akira S, **Klinman DM**, Belyakov IM, Berzofsky JA. Toll-like receptor ligands synergize through distinct dendritic cell pathways to induce T cell responses: implications for vaccines. PNAS. 105:16250, 2008.

264) Tross D, **Klinman DM.** Effect of CpG oligonucleotides on vaccine-induced B cell memory. J Immunology 181:5785, 2008.

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Vaccination with Novel Immunostimulatory Adjuvants against Blood-Stage Malaria in Mice

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An important aspect of malaria vaccine development is the identification of an appropriate adjuvant which is both capable of stimulating a protective immune response and safe for use by humans. Here, we investigated the feasibility of using novel immunostimulatory molecules as adjuvants combined with a crude antigen preparation and coadsorbed to aluminum hydroxide (alum) as a vaccine against blood-stage *Plasmodium chabaudi* AS malaria. Prior to challenge infection, immunization of genetically susceptible A/J mice with the combination of malaria antigen plus recombinant interleukin-12 (IL-12) in alum induced a Th1 immune response with production of high levels of gamma interferon (IFN-γ) and diminished IL-4 levels by spleen cells stimulated in vitro with parasite antigen compared to mice immunized with antigen alone, antigen in alum, or antigen plus IL-12. Mice immunized with malaria antigen plus recombinant IL-12 in alum had high levels of total malaria-specific antibody and immunoglobulin G2a. Compared to unimmunized mice, immunization with antigen plus IL-12 in alum induced the highest level of protective immunity against challenge infection with *P. chabaudi* AS, which was evident as a significantly decreased peak parasitemia level and 100% survival. Protective immunity was dependent on CD4$^+$ T cells, IFN-γ, and B cells and was long-lasting. Replacement of IL-12 as an adjuvant by synthetic oligodeoxynucleotides (ODN) containing CpG motifs induced a similar level of vaccine-induced protection against challenge infection with *P. chabaudi* AS. These results illustrate that it is possible to enhance the potency of a crude malaria antigen preparation delivered in alum by inclusion of immunostimulatory molecules, such as IL-12 or CpG-ODN.

Malaria vaccine development efforts during the past 20 years have been aimed at antigen identification, gene cloning, and expression of recombinant molecules (17). This has resulted in a number of promising blood-stage-derived recombinant antigens for inclusion in subunit vaccines, including MSP1, MSP2, MSP3, MSP4, MSP5, AMA1, PfEMP1, RESA, RAP1, and RAP2 (10, 18). Clinical trials with many of these candidates have been conducted or are ongoing (10, 13, 18). It is anticipated that the success of these trials may potentially lead to a vaccine capable of saving millions of lives each year from malaria.

An important aspect of vaccine development against infectious diseases, including malaria, is the identification of an appropriate adjuvant which is both capable of stimulating a protective immune response and safe for use by humans. Aluminum hydroxide, usually referred to as alum, which is approved as an adjuvant for use by humans, is not always the most appropriate adjuvant, given its potential to stimulate a Th2 type immune response characterized by immunoglobulin G1 (IgG1) and IgE production and the lack of induction of cytotoxic T-cell responses (5). This is particularly problematic in the development of vaccines against diseases caused by intracellular pathogens such as protozoan parasites, including intraerythrocytic *Plasmodium* parasites, the causative agent of malaria. Protective immunity against intracellular pathogens is generally dependent on Th1 type immune responses. However, protective immunity against blood-stage malaria is particularly complex and requires a concerted effort by a Th1 type cellular immune response and humoral immunity possibly involving a Th2 type response (24, 29). Rodent studies have revealed a role for CD4$^+$ T cells, B cells, and antibody in mediating naturally induced immunity against primary blood-stage infection (24, 29). The importance of CD4$^+$ T cells in immunity induced by vaccination with a defined antigen, such as MSP1, is less clear, although high titers of antigen-specific antibody correlate with protective immunity induced in mice by vaccination with the 19-kDa carboxyl-terminal fragment of MSP1 derived from *Plasmodium yoelii* (reviewed in reference 13).

Interleukin-12 (IL-12) plays an essential role in the differentiation of CD4$^+$ lymphocytes by promoting Th1 while suppressing Th2 cell development, thereby favoring gamma interferon (IFN-γ) production and elevated IgG2a levels (31). Because of its potent immunoregulatory properties, this cytokine has been used successfully as a vaccine adjuvant in models of intracellular infections, such as *Leishmania major* (1, 21) and *Listeria monocytogenes* (27), which require induction of Th1 responses for protective immunity. Consistent with the ability of IL-12 to promote a Th1-dependent immune response and to dampen a Th2 response, Wynn and colleagues (46–48) demonstrated that, as an adjuvant, IL-12 not only influences the promotion of protective Th1-dependent vaccine-induced immunity against *Schistosoma mansoni* but also prevents Th2-dependent pathology associated with this helminth parasite.

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Furthermore, as a vaccine adjuvant, IL-12 enhances both cell-mediated immune responses and augments antigen-specific IgG1, IgG2a, and IgG3 antibody levels, especially when the antigen and IL-12 are simultaneously coadsorbed to alum (19, 48).

In previous studies, our laboratory demonstrated that treatment of genetically susceptible A/J mice with exogenous IL-12 during primary *Plasmodium chabaudi* AS infection induces protective type 1 immunity, resulting in a less severe course of infection and survival (37). Studies with IL-12 p40-deficient mice confirmed the key role of IL-12 in inducing protective Th1 responses involving IFN-γ during the acute phase of infection and demonstrated that IL-12 is also important for antibody-mediated immunity during the chronic stage and in a challenge infection (39).

In the present study, we investigated the potential of using IL-12 as an adjuvant in a vaccine against blood-stage *P. chabaudi* AS malaria in A/J mice. Since our focus was to define the efficacy and characterize the protective immune mechanisms induced by the combination of an immunostimulatory molecule with malaria antigen, we used murine recombinant IL-12 together with a crude preparation of whole antigen from *P. chabaudi* AS parasitized red blood cells (PRBC). With this combination coadsorbed to alum, we report the induction of a protective antimalarial immune response in susceptible A/J mice characterized by the production of high levels of IFN-γ and parasite-specific IgG2a and protection against challenge infection with blood-stage *P. chabaudi* AS. Protective immunity was found to be dependent on $CD4^+$ T cells, B cells, and IFN-γ and to be long-lasting. Replacement of IL-12 as an adjuvant by synthetic oligodeoxynucleotides (ODN) containing CpG motifs was found to induce a similar level of vaccine-induced protection against challenge infection with *P. chabaudi* AS.

MATERIALS AND METHODS

Mice. Age- and sex-matched mice, 6 to 8 weeks old, were used in all experiments. A/J mice were purchased from the Jackson Laboratory (Bar Harbor, Maine) and C57BL/6 mice were purchased from Charles River Laboratories (St. Constant, Quebec, Canada). IFN-γ knockout (GKO) mice on the C57BL/6 background were bred in the animal facility of the Montreal General Hospital Research Institute from breeding pairs of GKO mice, which were originally from Genentech, Inc. (South San Francisco, Calif.) and backcrossed onto the C57BL/6 strain for eight generations by F. P. Heinzel (Case Western Reserve University School of Medicine, Cleveland, Ohio) (16). B-cell-deficient μ-MT with targeted disruption of the membrane exon of the immunoglobulin μ-chain gene or B-cell knockout (BKO) mice were originally derived on a 129 × C57BL/6 background and backcrossed to the C57BL/10 background for 12 generations (20, 22). B-cell-deficient μ-MT and wild-type C57BL/10SgSnAi mice were obtained from Taconic Farms, Inc. (Germantown, N.Y.).

$CD4^+$ T-cell depletion. Monoclonal anti-CD4 antibody from the hybridoma clone GK1.5 was raised as ascites fluid in BALB/c mice as previously described (33). The ascites fluid was delipified, dialyzed, and quantitated for the concentration of rat IgG. Mice were treated with the first dose of 500 μg of anti-CD4 antibody intraperitoneally (i.p.) 3 days prior to infection. Following infection, 200 μg was administered i.p. 3 times per week until the end of the experiment. Control mice received purified rat IgG at similar dosages and timing. Treatment with GK1.5 monoclonal antibody consistently depletes >98% of $CD4^+$ T cells based on fluorocytometric analysis (33, 37) and functional studies (33).

***P. chabaudi* AS infection and antigen preparation.** *P. chabaudi* AS was maintained as previously described (33). Naive and immunized mice were infected i.p. with 10^6 PRBC. The course and outcome of infection were monitored by previously described procedures (33). For the determination of cytokine and antibody levels in sera, mice were sacrificed at the indicated times and blood was obtained by cardiac puncture, allowed to clot for 30 min at 4°C, and centrifuged at 3,000 × *g* for 3 min. Sera were collected and stored at 4°C for measurement of IL-12 p70 or at −20°C for determination of the levels of other cytokines and malaria-specific antibodies.

Antigen was prepared by modification of a freeze-thaw protocol described by Amante and Good (2). Briefly, blood from A/J mice with parasitemias of 40 to 45% was collected, pooled, and centrifuged at 300 × *g* for 10 min. The red blood cell pellet was subjected to 2 rounds of lysis with distilled H_2O and centrifugation at 10,000 × *g* for 25 min. After 2 washes with phosphate-buffered saline (PBS), the parasite pellet was resuspended in PBS and subjected to 3 cycles of freeze-thaw at −70°C and 37°C. The suspension, containing both soluble and particulate antigens, was further disrupted by passage 2 to 3 times through a syringe with a 25-gauge needle.

Immunization protocol. An amount of malaria antigen equivalent to 1×10^7 to 1.5×10^7 PRBC was mixed with 1 μg of murine recombinant IL-12 (a kind gift from Wyeth Genetics Institute, Cambridge, Mass.) to a volume of 50 μl with PBS. An equal volume of alum (Imject alum; Pierce Chemical Co., Rockford, Ill.) was added, and the suspension was mixed thoroughly. Mice were immunized subcutaneously (s.c.) with 0.1 ml on the nape of the neck. Other groups of mice were also immunized in a similar manner with the following vaccine combinations: antigen suspended in PBS, antigen admixed in alum, and antigen admixed with 1 μg of murine recombinant IL-12 in PBS. Three weeks later, the antigen-treated groups were boosted with the same amount of antigen in 0.1 ml of PBS injected i.p. Mice were challenged i.p. with 10^6 PRBC 2 weeks later.

CpG DNA. ODN containing CpG motifs (CpG-ODN no. 1826) and control ODN (no. 1982) were a kind gift of Coley Pharmaceuticals Canada (Ottawa, Ontario, Canada). One hundred micrograms of CpG-ODN or control ODN was admixed with antigen and alum and used according to the standard immunization protocol described above.

Spleen cell culture and proliferation assay. Spleens from immunized mice were removed aseptically and pressed through a sterile fine-wire mesh with 10 ml of RPMI 1640 (Gibco-Invitrogen, Burlington, Ontario, Canada) supplemented with 5% heat-inactivated fetal calf serum (HyClone Laboratories, Logan, Utah), 25 mM HEPES (Gibco-Invitrogen), 0.12% gentamicin (Schering, Montreal, Quebec, Canada), and 2 mM glutamine (Gibco-Invitrogen). Cell suspensions were centrifuged at 350 × *g* for 10 min. Red blood cells were lysed with 0.175 M NH_4Cl, and the cells were washed twice in fresh medium. Membrane debris was removed by filtering the cell suspensions through sterile gauze. The viability of the cells was determined by trypan blue exclusion and was always >90%. Total cell counts were performed on individual samples. For proliferation assays, spleen cells were adjusted to 2.5×10^6 cells/ml and aliquots of 0.1 ml were plated in triplicate in 96-well flat-bottom plates, stimulated with 10^6 washed PRBC/ml (as the malaria parasite antigen) or medium (as the control), and incubated for 72 h at 37°C in a humidified CO_2 incubator. During the last 16 h of culture, 1 μCi of [^{3}H]thymidine (specific activity, 6.7 Ci/mmol) was added to each well, the cells were harvested with an automatic cell harvester, and the incorporated radioactivity was measured in a liquid scintillation counter. For determination of cytokine production, spleen cells were adjusted to 5×10^6 cells/ml and aliquots of 1 ml were plated in triplicate in 24-well tissue culture plates in the presence or absence of 10^6 PRBC, as described above, and incubated for 48 h at 37°C in a humidified CO_2 incubator. Supernatants were collected, centrifuged at 350 × *g* for 5 min, and stored at 4°C or at −20°C until assayed for cytokine levels.

Cytokine ELISAs. Cytokine levels in sera and spleen cell supernatants were measured by using two-site sandwich enzyme-linked immunosorbent assays (ELISAs) for IFN-γ and tumor necrosis factor alpha (TNF-α) as previously described (34, 37). For IL-4, the capturing and detecting antibodies were BVD4-1D11 monoclonal antibody (MAb) and biotinylated BVD6-24G2 MAb, respectively. For IL-10, JES5.2A5 MAb (American Type Culture Collection, Rockville, Md.) and biotinylated SXC-1 MAb (BD Bioscience, Mississauga, Ontario, Canada) were used as the capturing and detecting antibodies, respectively. Standard curves for each cytokine were generated by using recombinant cytokines (BD Bioscience). Reactivity was revealed by using 2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid) (ABTS) substrate (Roche, Laval, Quebec, Canada) and optical density (OD) values were read in a microplate reader at 405 nm, with a reference wavelength of 492 nm.

Malaria-specific antibody ELISA. Serum levels of *P. chabaudi* AS-specific antibody isotypes were determined by ELISA. *P. chabaudi* AS antigen was prepared as described previously (49). Immulon II plates (Dynatech, Chantilly, Va.) were coated with parasite antigen at a concentration of approximately 4 to 5 μg/ml in PBS based on an OD at 280 nm overnight at 4°C and subsequently blocked with 1% bovine serum albumin in PBS for 1 h. Individual serum samples were serially diluted twofold, and 50 μl of each dilution was added to the plate and incubated for 2 h at room temperature. The data shown are based on values obtained at the following dilutions: total Ig, 1:20; IgG1, 1:10; IgG2a, 1:10. After extensive washing, horseradish peroxidase-conjugated goat anti-mouse isotype

TABLE 1. Antigen-specific spleen cell proliferation and cytokine responses in immunized mice prior to *P. chabaudi* AS challenge infection

Treatment group[a]	Antigen proliferation (cpm ± SEM)	Level of cytokine (Mean ± SEM):			
		IFN-γ (ng/ml)	TNF-α (pg/ml)	IL-4 (pg/ml)	IL-10 (pg/ml)
Untreated	526 ± 71	0.39 ± 0.39	98.41 ± 10.22	254.27 ± 52.80	0.76 ± 0.01
Antigen	486 ± 44	5.13 ± 0.36	122.03 ± 17.78	178.55 ± 45.13	1.07 ± 0.11
Antigen + alum	1,162 ± 128	3.83 ± 0.86	175.03 ± 20.93	234.52 ± 20.60	1.27 ± 0.10
Antigen + IL-12	768 ± 130	11.14 ± 2.27	189.85 ± 28.58	134.19 ± 33.22	1.01 ± 0.09
Antigen + IL-12 + alum	2,768 ± 622[b]	43.13 ± 5.20[c]	341.94 ± 44.26[c]	165.87 ± 42.82[d]	1.44 ± 0.14[e]

[a] Groups of A/J mice (5 per group) were immunized s.c. with malaria antigen alone, antigen in alum, antigen plus 1.0 μg of IL-12, or antigen plus 1.0 μg of IL-12 in alum and boosted 3 weeks later by i.p. injection with antigen. Data from one of two replicate experiments are presented.
[b] $P < 0.05$ for antigen plus alum versus antigen plus IL-12 plus alum.
[c] $P < 0.0001$ for antigen plus alum versus antigen plus IL-12 plus alum.
[d] $P < 0.01$ for antigen plus alum versus antigen plus IL-12 plus alum.
[e] $P < 0.008$ for antigen plus alum versus antigen plus IL-12 plus alum.

antibodies (SBA, Birmingham, Ala.) were added and incubated at room temperature for another 2 h. Reactivity was visualized with ABTS substrate, and OD values were read in a microplate reader at 405 nm, with a reference wavelength of 492 nm. Antibody levels in serum are expressed as relative OD.

Statistical analysis. Data are presented as means ± standard errors of the means. The statistical significance of differences in means between experimental and control groups was analyzed by Student's *t* test with SAS/STAT software (SAS Institute, Cary, N.C.). A *P* value of <0.05 was considered significant.

RESULTS

Immunization with malaria antigen plus IL-12 in alum induces a Th1 immune response. Since a strong Th1 immune response is associated with protective immunity to acute blood-stage *P. chabaudi* AS during a primary infection, the type of immune response induced by inclusion of IL-12 in a vaccine formulation was first evaluated. *P. chabaudi* AS susceptible A/J mice were immunized s.c. with a freeze-thaw preparation of blood-stage malaria antigen alone, antigen in alum, antigen plus IL-12, or antigen plus IL-12 in alum and boosted 3 weeks later by i.p. injection with antigen alone. Two weeks later, prior to challenge infection, immunized mice and untreated, control A/J mice were sacrificed and proliferation and cytokine production by spleen cells were analyzed in vitro. As shown in Table 1, immunization with either antigen in alum or the combination of antigen plus IL-12 in alum resulted in significantly increased antigen-specific proliferation compared to the response of control A/J mice ($P = 0.02$ and $P = 0.037$, respectively). However, the combination of antigen plus IL-12 in alum resulted in a greater than twofold increase in proliferation compared to antigen in alum, which represents a significant difference between the two groups. Furthermore, in comparison with spleen cells from mice immunized with antigen in alum, spleen cells from mice immunized with the combination of antigen plus IL-12 in alum produced significantly higher levels of the Th1 cytokines, IFN-γ and TNF-α, and significantly lower levels of IL-4. Spleen cells from mice immunized with the combination of antigen plus IL-12 in alum also produced modest levels of IL-10, which were significantly higher than the response of cells from mice immunized with antigen in alum.

The levels of total malaria-specific antibody and IgG1 and IgG2a in the sera of immunized A/J mice were also analyzed 2 weeks after boosting prior to challenge infection. Total malaria-specific antibody was significantly and similarly increased in the three groups of immunized animals compared with the levels of total specific antibody in mice immunized with antigen alone (Fig. 1A). Malaria-specific IgG1 was significantly increased in the groups immunized with antigen in alum and the combination of antigen plus IL-12 in alum compared to IgG1 levels in mice immunized with antigen alone (Fig. 1B). However, the level of malaria-specific IgG1 was significantly higher in the group immunized with antigen in alum than in those immunized with the combination of antigen plus IL-12 in alum. The levels of specific IgG2a were significantly increased compared to controls only in mice immunized with the combination of antigen plus IL-12 in alum (Fig. 1C). These findings demonstrate that immunization with the combination of malaria antigen plus IL-12 in alum induced high levels of production of the Th1 cytokine IFN-γ and parasite-specific IgG2a. In addition, mice immunized with this combination produced significantly lower levels of antigen-specific IL-4 and IgG1 than did mice immunized with antigen in alum in the absence of IL-12.

Immunization with malaria antigen plus IL-12 in alum induces protection against challenge infection with blood-stage *P. chabaudi* AS. To compare the efficacy of vaccination with the various combinations in conferring protective immunity, groups of A/J mice, immunized as described above, were challenged i.p. with *P. chabaudi* AS 2 weeks after boosting and the course of parasitemia and the outcome of infection were monitored. Similar to control mice, mice immunized with antigen alone or antigen in alum suffered a severe course of parasitemia with high peak parasitemia levels and high mortality (Fig. 2A and C). Mice immunized with antigen plus IL-12 or antigen plus IL-12 in alum experienced less severe courses of infection with significantly lower peak parasitemia levels than control mice ($P < 0.001$ and $P < 0.001$, respectively) (Fig. 2B). In the case of mice immunized with antigen plus IL-12 in alum, there was a delay of 1 to 2 days in peak parasitemia level compared to nonimmunized mice. Although antigen plus IL-12 was effective in significantly reducing peak parasitemia compared to control mice, only 60% (9 of 15) of mice immunized with this combination survived while 100% (25 of 25) of mice immunized with the combination of antigen plus IL-12 in alum survived challenge infection with *P. chabaudi* AS. These results indicate that antigen plus IL-12 in alum was the best combination for conferring protection against blood-stage malaria in terms of reduced parasitemia and enhanced survival.

Immunization with malaria antigen plus IL-12 in alum induces long-lasting protection. An important characteristic of

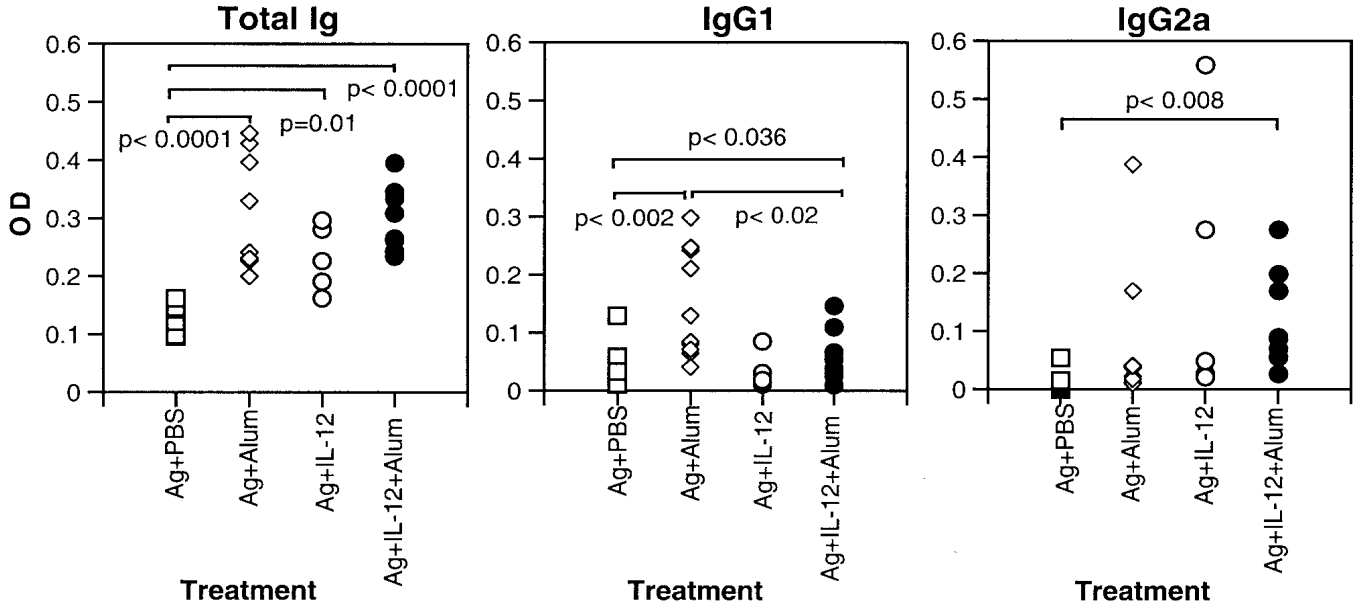

FIG. 1. Levels of malaria-specific antibodies in the sera of A/J mice immunized s.c. with antigen (Ag) alone, antigen in alum, antigen plus IL-12, or antigen plus IL-12 in alum and boosted 3 weeks later by i.p. injection with antigen. Two weeks later, sera were collected from immunized mice and the levels of total malaria-specific antibody, IgG1, and IgG2a were determined by ELISA. Data represent OD values for individual mice and are pooled from 2 experiments.

an effective malaria vaccine is that the elicited immunity is long-lasting. To address this issue, A/J mice were immunized with the combination of antigen plus IL-12 in alum and challenged as before, that is, 2 weeks after boosting, or 12 weeks after boosting. Similar to mice challenged 2 weeks after boosting, A/J mice challenged at 12 weeks were solidly immune (Table 2). Long-lasting protection induced in these animals by malaria antigen plus IL-12 in alum was evident by a number of parameters. Importantly, there was a significant decrease in peak parasitemia compared to nonimmunized A/J mice ($P < 0.001$). In addition, the number of days required to clear parasites from the blood of mice challenged 12 weeks after boosting was similar to that for mice challenged 2 weeks after boosting, and there was 100% survival among all immunized mice regardless of the time of challenge infection.

Protective immunity induced by immunization with malaria antigen plus IL-12 in alum requires CD4$^+$ T cells and IFN-γ. To investigate the mechanism of protective immunity induced by vaccination with the combination of antigen plus IL-12 in alum, immunized A/J mice were depleted of CD4$^+$ T cells by treatment with GK1.5 MAb 3 days prior to and three times per week during the challenge infection with *P. chabaudi* AS. Parasitemia and survival were monitored for 4 weeks post-challenge infection. Consistent with the results shown above, intact immunized A/J mice suffered a mild course of infection and survived challenge infection. In contrast, CD4$^+$-T-cell-depleted mice experienced fulminant infections with significantly higher peak parasitemia levels than rat IgG-treated mice ($P = 0.008$) (Fig. 3A), and the animals died by day 11 postchallenge.

To determine the role of IFN-γ in vaccine-induced protection, GKO mice on the resistant C57BL/6 background and wild-type C57BL/6 mice (36) were immunized with antigen plus IL-12 in alum. Immunized as well as untreated, control GKO and wild-type mice were challenged with *P. chabaudi* AS as described above. The course of parasitemia and outcome of infection were monitored for 4 weeks in control and immunized mice of both genotypes (Fig. 3B and C). As shown previously, control GKO mice developed significantly higher levels of peak parasitemia on day 7 than their wild-type counterparts (64.2 ± 3.35 versus 38.7 ± 4.43, respectively; $P < 0.0001$). Furthermore, immunized wild-type C57BL/6 mice had a significantly lower peak parasitemia level which occurred 1 day later than that for wild-type mice without immunization ($P < 0.0001$) (Fig. 3B), indicating that immunization with antigen plus IL-12 in alum induced protection in resistant C57BL/6 as well as susceptible A/J hosts. In contrast to increased protection, as defined by the level of peak parasitemia, observed in wild-type mice, there was no significant difference in peak parasitemia levels in immunized versus untreated GKO mice (55.31 ± 1.37 versus 64.2 ± 3.35, respectively; $P = 0.05$). The timing of the peak parasitemia was delayed from day 7 to day

TABLE 2. Long-term protection against blood-stage malaria induced by immunization with malaria antigen plus IL-12 in alum

Treatment group[a] (*n*)	% Peak parasitemia (mean ± SEM)	Clearance by day:	% Survival
Untreated (10)	41.25 ± 1.29		0
2 wk postboost (5)	15.80 ± 2.29[b]	14	100
12 wk postboost (5)	28.65 ± 1.29[c]	15	100

[a] Groups of A/J mice ($n = 5$) were immunized s.c. with antigen plus 1.0 μg of IL-12 admixed in alum and boosted 3 weeks later by i.p. injection with antigen. For each immunization group, age-matched untreated and immunized mice were infected i.p. with 10^6 PRBC at 2 or 12 weeks postboost. Since there were no significance differences in peak parasitemia or survival between the two untreated groups ($n = 10$), data have been pooled.

[b] $P < 0.001$ compared to control.

[c] $P < 0.001$ compared to control.

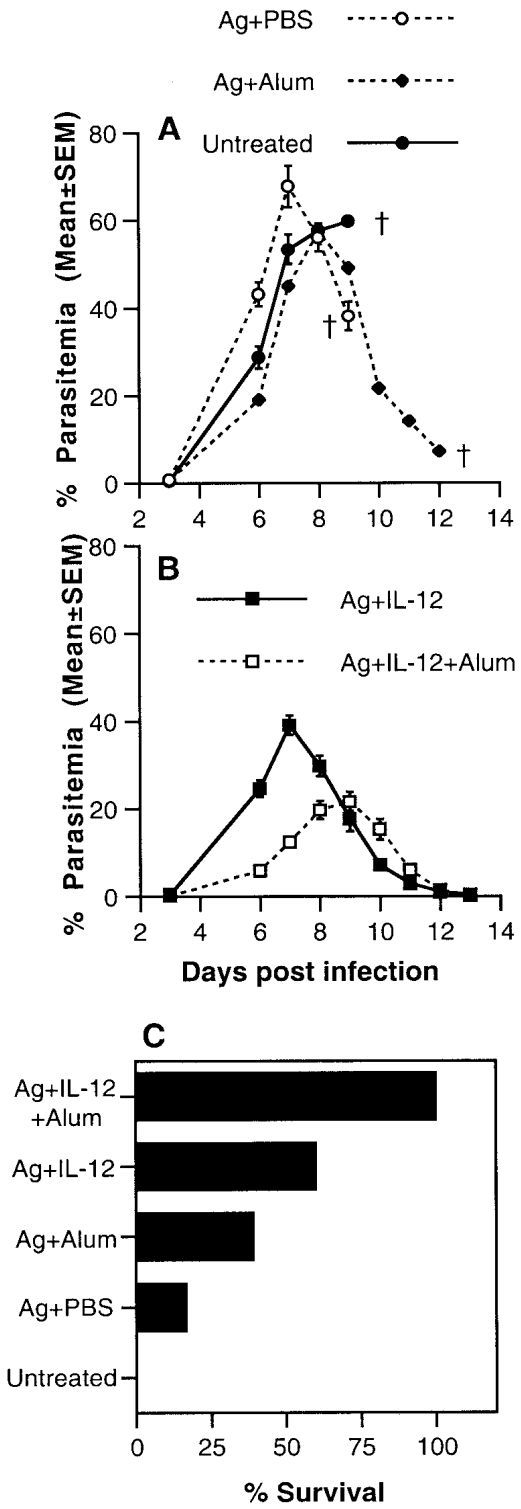

FIG. 2. Course of parasitemia and survival in A/J mice immunized s.c. with antigen (Ag) alone, antigen in alum, antigen plus IL-12, or antigen plus IL-12 in alum and boosted 3 weeks later by i.p. injection with antigen. Two weeks later, immunized and untreated control mice were challenged i.p. with 10^6 *P. chabaudi* AS PRBC. The percentage of PRBC in peripheral blood (A and B) was determined for each group of 5 mice. Data from one of two replicate experiments are presented. Mice were examined twice daily for the duration of the experiment for survival (C). Cumulative data from 6 experiments are presented.

9 in immunized mice compared to control GKO mice. However, 100% of GKO mice, whether immunized or not, succumbed to challenge infection by day 12 (40; data not shown). Taken together, these results demonstrate the crucial roles of $CD4^+$ T cells and IFN-γ in the development of protective immunity against blood-stage malaria induced by immunization with *P. chabaudi* AS antigen plus IL-12 coadsorbed to alum.

Protective immunity induced by immunization with malaria antigen plus IL-12 in alum requires B cells. As shown above, immunization of A/J mice with malaria antigen plus IL-12 in alum induced high levels of total malaria-specific antibody, IgG2a, and IgG1, and conferred the highest level of protection against challenge infection with blood-stage *P. chabaudi* AS. These observations suggested to us that the B-cell response is an integral component of the mechanism of protective immunity induced by immunization with the combination of malaria antigen and IL-12 coadsorbed to alum. The role of B cells in protective immunity induced by vaccination with antigen plus IL-12 in alum was further investigated by using B-cell-deficient μ-MT mice on the resistant C57BL/10 background (36). As previously observed (41, 43, 44), nonimmunized male (Fig. 4A) and female (Fig. 4C) B-cell-deficient mice compared to intact C57BL/10 mice (Fig. 4B and D) experienced recurrent bouts of recrudescent parasitemia until the experiment was terminated 90 days after challenge infection. Following immunization, peak parasitemia levels in male and female intact C57BL/10 mice were significantly decreased ($P < 0.001$ for male mice and $P < 0.05$ for female mice). Challenge infection was cleared in both male and female immunized C57BL/10 mice, although female mice experienced several recrudescent parasitemias between 5 and 10%. Despite immunization, male and female B-cell-deficient mice experienced peak parasitemias which were not significantly reduced compared to nonimmunized, B-cell-deficient mice. Although immunized B-cell-deficient mice suffered fewer and significantly lower recrudescent parasitemias than their nonimmunized counterparts, they were unable to clear the infection completely and low levels of parasitemia (1 to 5%) persisted throughout the chronic stage of infection until the experiment was terminated on day 90.

CpG-ODN can replace IL-12 as an adjuvant for immunization against blood-stage malaria. It is possible that other agents, such as CpG-ODN, with potent immunostimulatory properties could also be useful as an adjuvant in a vaccine against blood-stage malaria. CpG-ODN has been shown to induce production of IL-12, which, in turn, enhances IFN-γ production, antibody production by B cells, and cytotoxicity of NK cells and $CD8^+$ T cells (4, 5, 7, 15, 23). To determine whether CpG-ODN can replace IL-12 as an adjuvant in the blood-stage malaria vaccine, A/J mice were immunized with malaria antigen plus 100 μg of CpG-ODN or control ODN in alum, by using the standard protocol, and challenged with *P. chabaudi* AS. As shown in Fig. 5, CpG-ODN was as effective as IL-12 in inducing protection against challenge infection with *P. chabaudi* AS. Mice immunized with malaria antigen plus CpG-ODN in alum had a course of parasitemia and 100% survival following challenge infection with *P. chabaudi* AS, which was similar to mice immunized with antigen plus IL-12 in alum. There was a significant decrease in the peak parasitemia levels of mice immunized with antigen plus CpG-ODN in alum com-

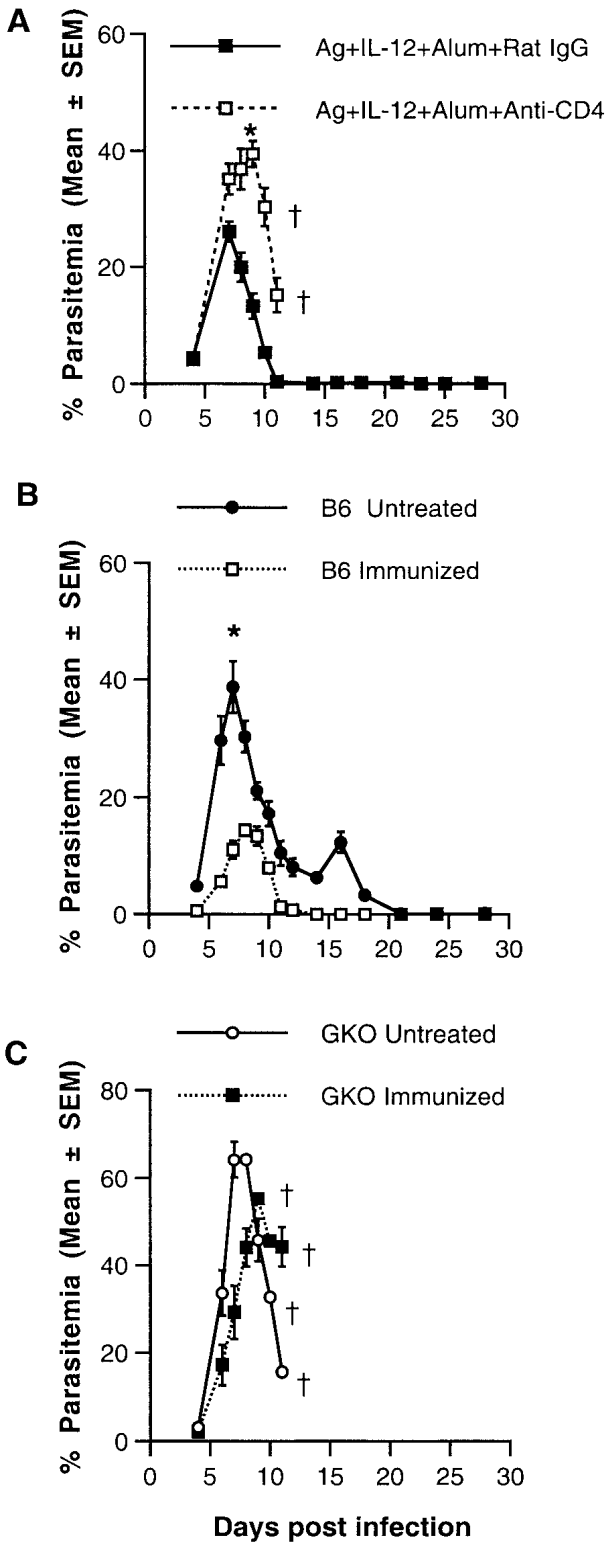

FIG. 3. Course of parasitemia and survival in immunized $CD4^+$ T cell depleted A/J mice or in wild-type or GKO C57BL/6 mice. To deplete $CD4^+$ in vivo, A/J mice were treated i.p. with GK1.5 monoclonal antibody or with an equivalent amount of rat IgG as a control 3 days prior to challenge infection and three times per week during infection. Two weeks after boosting, mice were challenged i.p. with 10^6 *P. chabaudi* AS PRBC and the course of parasitemia was determined (A). Female wild-type (B) and GKO (C) C57BL/6 mice were immunized with antigen (Ag) plus IL-12 in alum, and 2 weeks after boosting, mice were challenged i.p. with 10^6 *P. chabaudi* AS PRBC and the course of parasitemia was determined. Similar results were obtained in a replicate experiment using male wild-type and GKO mice. In panel A, the asterisk designates a P value of <0.001 for control versus $CD4^+$-T-cell-depleted mice. In panel B, the asterisk designates a P value of <0.0001 for untreated versus immunized C57BL/6 mice.

pared to mice immunized with antigen plus control ODN in alum ($P < 0.001$), and mice in the former group cleared the parasite by 2 weeks postinfection. The combination of antigen plus control ODN in alum was not protective, and 100% of the mice in this group succumbed to challenge infection, with fulminant parasitemia levels by day 10 postinfection.

DISCUSSION

Previous studies demonstrate that coadsorption of antigen and IL-12 to alum promotes both type 1 cytokine and antibody responses (19, 21). Since both cellular and humoral responses have been implicated in protective immunity to malaria, we reasoned that immunization with the combination of malaria antigen and IL-12 coadsorbed to alum may enhance protective immunity to blood-stage malaria. To investigate this possibility, we examined the feasibility of using crude malaria antigen coadsorbed with IL-12 to alum as a vaccine against blood-stage malaria in the mouse model of *P. chabaudi* AS. Cellular and humoral immune responses were compared in A/J mice immunized with antigen plus IL-12 in alum as well as antigen alone, antigen in alum, or antigen plus IL-12 and boosted 3 weeks later with antigen alone prior to challenge infection.

A/J mice are susceptible to primary *P. chabaudi* AS infection and experience fulminant and lethal parasitemia by 10 to 13 days postinfection (36). During the first week of infection, spleen cells from these mice produce high levels of IL-4 and low levels of IFN-γ in vitro in response to the parasite antigen (38). Determination of proliferation and cytokine production in vitro by spleen cells from A/J mice immunized with the various vaccine combinations revealed that spleen cells from mice immunized with malaria antigen plus IL-12 in alum had the highest levels of proliferation as well as of IFN-γ production in response to the specific antigen. Spleen cells from these mice also produced lower levels of the Th2 cytokine IL-4 and the Th1 cytokine TNF-α and low levels of IL-10.

The finding of high levels of production of IFN-γ is consistent with observations in previous studies with IL-12 as an adjuvant together with antigen for vaccination against *L. major* (1), *S. mansoni* (46–48), or *Listeria* (27), which demonstrate that inclusion of this cytokine in the vaccine formulation induces a strongly polarized type 1 cytokine response with production of high levels of IFN-γ. In these studies, the combination of IL-12 and antigen was administered as a vaccine in the absence of alum or another adjuvant. However, Jankovic et al. (19) observed that administration of IL-12 and human immunodeficiency virus type 1 gp120 induces a shift from a type 2 to a type 1 cytokine profile only when coadsorbed to alum. In the case of vaccination against *L. major* (1) or *S. mansoni* (46–48) with the combination of parasite antigen and IL-12, Th2 cytokine production, including IL-4 production, is markedly diminished. Taken together, these findings in various infection mod-

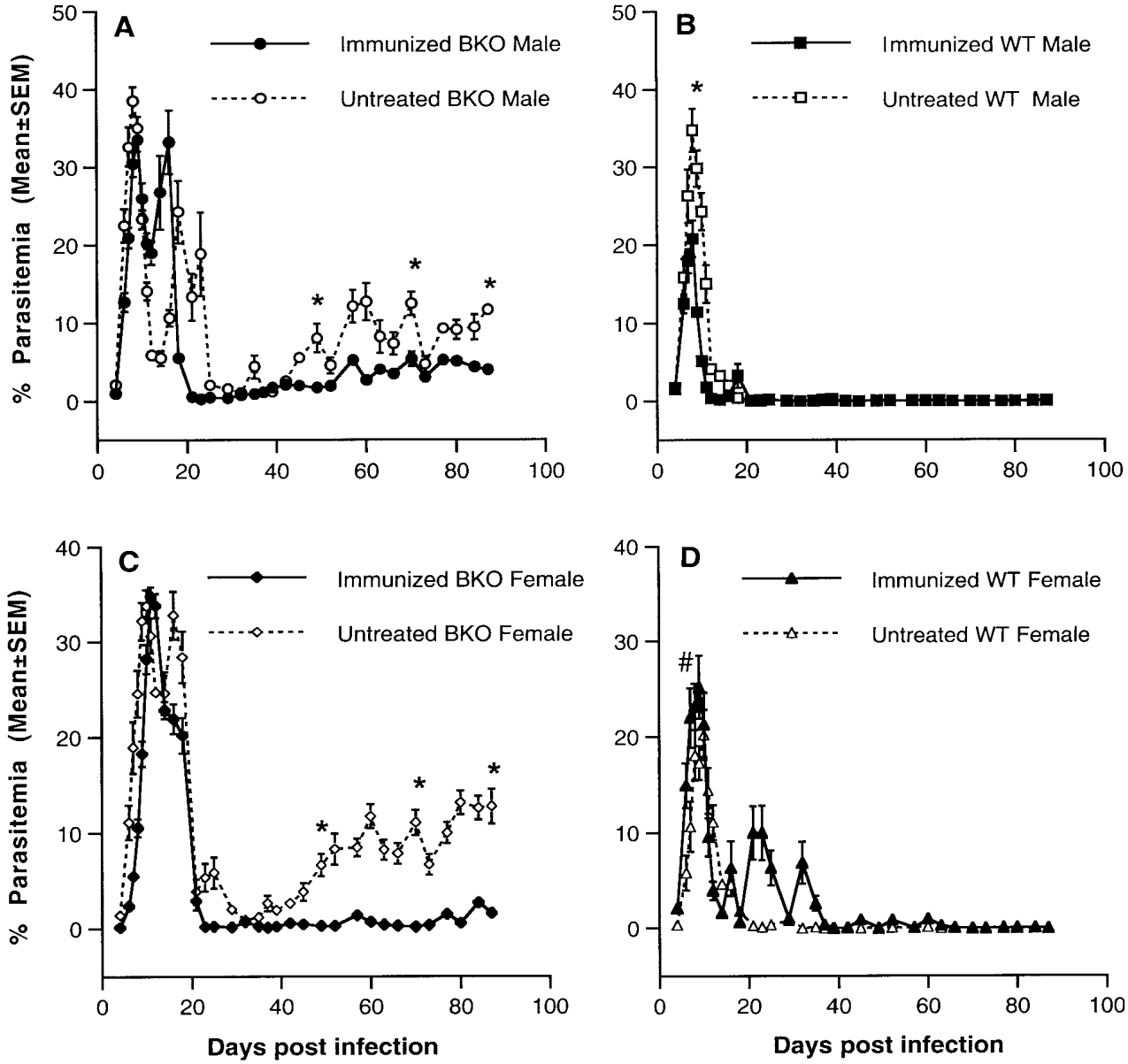

FIG. 4. Course of parasitemia in immunized B-cell-deficient μ-MT (BKO) and wild-type (WT) C57BL/10 mice. Groups of BKO (male, n = 6; female, n = 8) and WT (male and female, n = 10) mice were immunized s.c. with antigen plus IL-12 in alum and boosted i.p. with antigen 3 weeks later. Two weeks later, mice were challenged i.p. with 10^6 *P. chabaudi* AS PRBC and the course of parasitemia was determined in male (A and B) and female (C and D) BKO (A and C) and wild-type (B and D) mice. *, $P < 0.001$; #, $P < 0.05$ for nonimmunized versus immunized mice.

els with mice are in accordance with the ability of IL-12 to promote the differentiation of $CD4^+$ Th1 cells (12).

Our observations that the combination of malaria antigen plus IL-12 in alum induced high levels of total malaria-specific antibody and IgG2a antibody as well as moderate levels of malaria-specific IgG1 are consistent with previous studies utilizing IL-12 in a vaccine formulation. The present study did not address whether the increased protection seen in the group of mice immunized with the combination of malaria antigen plus IL-12 in alum is due to a particular subclass or isotype of antibody or due to a generalized increase in antibody titers. Wynn et al. (48) demonstrated that mice vaccinated with a combination of IL-12 and irradiated *S. mansoni* cercariae have significant increases in parasite-specific IgG2a, IgG2b, and IgG1. Studies by Jankovic et al. (19) demonstrated that mice vaccinated with IL-12 and recombinant gp120 envelope protein from human immunodeficiency virus type 1 coadsorbed to alum have high levels of specific IgG2a, IgG2b, and IgG3 isotypes as well as significantly increased gp120-specific IgG1 isotype levels compared to mice immunized with antigen in alum. Interestingly, in these two studies, enhanced IgG1 production occurred in the face of suppressed IL-4 production when IL-12 was included in the vaccine formulation. Similarly, our results indicate that vaccination with the combination of malaria antigen plus IL-12 coadsorbed to alum induced a Th1 immune response in vaccinated mice. The induction of a Th1

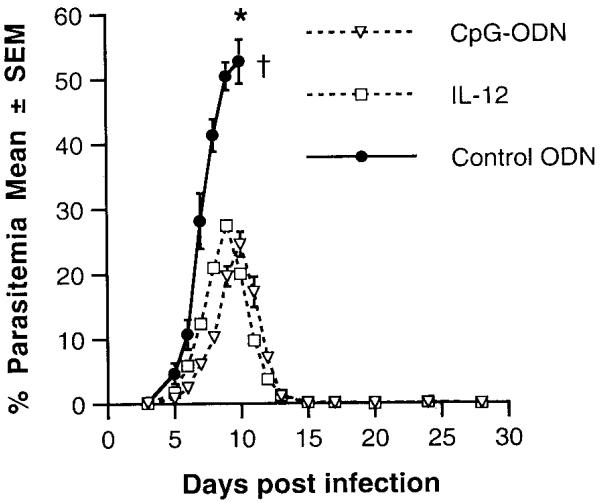

FIG. 5. Course of parasitemia in A/J mice immunized with antigen plus IL-12 in alum or antigen plus CpG-ODN in alum. Groups of 5 A/J mice were immunized s.c. with either antigen plus IL-12 in alum (IL-12), antigen plus CpG-ODN in alum (CpG-ODN), or antigen plus ODN in alum (Control ODN) and boosted i.p. with antigen 3 weeks later. Two weeks later, mice were challenged i.p. with 10^6 *P. chabaudi* AS PRBC and the course of parasitemia was determined. *, $P < 0.001$ for day 9 parasitemia between antigen plus ODN in alum versus antigen plus CpG-ODN in alum and $P = 0.114$ for antigen plus CpG-ODN in alum versus antigen plus IL-12 in alum.

immune response by administration of malaria antigen plus IL-12 coadsorbed to alum is relevant given the important role of type 1 cell-mediated and humoral immune responses in mediating naturally induced immunity against malaria in mice infected with blood-stage *P. chabaudi* AS and possibly in humans (24, 29, 39, 40).

Importantly, immunization with the combination of malaria antigen plus IL-12 in alum induced strong protective immunity against challenge infection with blood-stage *P. chabaudi* AS in both susceptible A/J and resistant C57BL/6 mice. In contrast to control A/J mice, which experience a severe course of parasitemia and 100% mortality (36), immunization with either antigen plus IL-12 or antigen plus IL-12 coadsorbed to alum resulted in less severe courses of infection and significant decreases in peak parasitemia levels. However, only mice immunized with antigen plus IL-12 in alum experienced 100% survival. Moreover, the protection induced by this formulation was long-lasting since mice challenged 3 months after boosting were still completely protected against *P. chabaudi* AS. This group of animals had significant decreases in peak parasitemia levels and time to parasite clearance comparable to mice challenged 2 weeks after boosting. In both instances, there was 100% survival of vaccinated mice.

Although $CD4^+$ T cells are known to play an important role in immunity to primary blood-stage *P. chabaudi* AS (24, 29), little is known about the role of these cells in vaccine-induced immunity to blood-stage malaria. Earlier studies by Langhorne and colleagues (25) demonstrated that depletion of $CD4^+$ T cells from immune C57BL/6 mice results in a low, transient parasitemia following challenge with *P. chabaudi* AS, which is eventually cleared. In contrast, our results in $CD4^+$-T-cell-depleted, immunized mice indicate that $CD4^+$ T cells play a critical role in immunity induced by vaccination with malaria antigen and IL-12 in alum. We observed that immunized $CD4^+$-T-cell-depleted mice experienced severe and lethal infections when challenged with *P. chabaudi* AS.

It is likely that $CD4^+$ T cells participate in immunity induced by immunization with malaria antigen and IL-12 coadsorbed to alum by producing IFN-γ, although the studies performed here did not address the cellular source of this cytokine in immunized mice. Both $CD4^+$ T cells and NK cells were found to produce IFN-γ in mice vaccinated with *L. major* antigen and IL-12 (1). NK cells may also be a source of IFN-γ in mice immunized with malaria antigen and IL-12 in alum. NK cells have been found to produce IFN-γ early in infection with various species of mouse malaria parasites, including *P. chabaudi* AS (8, 28). Recent studies with humans demonstrated that *Plasmodium falciparum*-infected red blood cells induce IFN-γ production by NK cells from individuals infected with *P. falciparum* and nonexposed donors (3). IFN-γ is considered to be a major component of innate and acquired immunity to primary blood-stage *P. chabaudi* infections (11, 24, 40, 42). The inability to protect GKO mice, compared to wild-type C57BL/6 mice, against challenge infection as shown here indicates that IFN-γ is also a critical cytokine in vaccine-induced immunity following immunization with malaria antigen and IL-12 coadsorbed to alum. In humans, IFN-γ production has been found to correlate with resistance to reinfection with *P. falciparum* as well as with protection from clinical attacks of malaria (6, 9, 26). Based on these observations, it has been concluded that IFN-γ production should be considered an important hallmark of effector T-cell function for the development of an effective malaria vaccine (14, 32). Our results in the present report support this contention.

During primary *P. chabaudi* AS infection, mice rendered B-cell deficient by treatment from birth with anti-IgM antibodies or μ-MT mice with targeted disruption of the membrane exon of the immunoglobulin μ-chain gene can control acute parasitemias in a manner similar to that of intact mice (41, 44). However, B-cell-deficient mice maintain a chronic low level of parasitemia, indicating that effective parasite clearance at the later, chronic stage of infection requires the presence of B cells (41, 44). In addition to their ability to produce antibody, B cells may also play a role via production of IL-10 (41) in the switch from Th1 cells producing IFN-γ, which mediates control of acute parasitemia, to Th2 cells which provide help for antibody production leading to clearance of primary blood-stage *P. chabaudi* AS infection. Studies in μ-MT mice also showed that B-cell-deficient animals are unable to control a challenge infection and develop parasitemia levels similar in magnitude to a primary infection (44). These findings suggest that B-cell-dependent mechanisms may be important for an effective memory response to *P. chabaudi* AS infection (44). In the present study, we observed that immunization of B-cell-deficient μ-MT mice with malaria antigen and IL-12 coadsorbed to alum is ineffective in providing enhanced protection against challenge infection with *P. chabaudi* AS, suggesting a role for a B-cell-dependent mechanism(s) in vaccine-induced immunity.

We also examined the possibility of replacing IL-12 with immunostimulatory CpG-ODN. Because of its ability to induce a type 1 pattern of cytokine production dominated by IL-12

and IFN-γ with little secretion of type 2 cytokines, CpG-ODN have been found to be useful as adjuvants for vaccines, including peptide vaccines, against a variety of pathogens (4, 5, 7, 15, 23, 30, 35, 45). Near and colleagues (30) recently demonstrated that vaccination with the combination of CpG-ODN and *P. yoelii* $MSP1_{19}$ in alum resulted in a dramatic elevation of IFN-γ production as well as elevated production of IL-10 by $MSP1_{19}$-stimulated splenocytes, suggesting induction of a mixed Th1 and Th2 response. In mice vaccinated with this formulation, IgG1 was found to be the predominant antibody isotype in sera, although increased levels of $MSP1_{19}$-specific IgG2a, IgG2b, and IgG3 isotype antibodies were also observed. Furthermore, increased antibody levels were found to correlate with protection against challenge infection with a high dose of *P. yoelii* PRBC. Our experimental results demonstrate that inclusion of immunostimulatory CpG-ODN instead of IL-12 in the vaccine formulation provided strong protection against blood-stage *P. chabaudi* AS infection in A/J mice. Studies in progress in our laboratory demonstrate that immunization with CpG-ODN and crude malaria antigen in alum induces high levels of malaria-specific IgG2a in A/J mice before challenge infection in comparison to immunization with control ODN and antigen in alum (unpublished data).

In conclusion, the results of this study illustrate that it is possible to enhance the potency of a crude malaria antigen in alum vaccine formulation by inclusion of agents with immunostimulatory properties, such as IL-12 or CpG-ODN. Although alum is the most commonly used adjuvant and is approved for use by humans by the U.S. Food and Drug Administration, our data indicate that this adjuvant is weak in promoting vaccine-induced protective immunity against blood-stage malaria. Furthermore, immunity induced by immunization with malaria antigen and IL-12 coadsorbed to alum induced a long-lasting, Th1 immune response required for protection against challenge infection with *P. chabaudi* AS infection.

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REFERENCES

1. **Alfonso, L. C. C., T. M. Scharton, L. Q. Vieira, M. Wysocka, G. Trinchieri, and P. Scott.** 1994. The adjuvant effect of interleukin-12 in a vaccine against *Leishmania major.* Science **263:**235–237.
2. **Amante, F. H., and M. F. Good.** 1997. Prolonged Th1-like response generated by a *Plasmodium yoelii*-specific T cell clone allows complete clearance of infection in reconstituted mice. Parasite Immunol. **19:**111–126.
3. **Artavanis-Tsakonas, K., and E. M. Riley.** 2002. Innate immune response to malaria: rapid induction of IFN-γ from human NK cells by live *Plasmodium falciparum*-infected erythrocytes. J. Immunol. **169:**2956–2963.
4. **Braziolot-Millan, C. L., R. Weeratna, A. M. Krieg, C. A. Siegrist, and H. L. Davis.** 1998. CpG DNA can induce strong Th1 humoral and cell-mediated immune responses against hepatitis B surface antigen in young mice. Proc. Natl. Acad. Sci. USA **95:**1555–1558.
5. **Davis, H. L., R. Weeratna, T. J. Waldschmidt, L. Tygrett, J. Schorr, and A. M. Krieg.** 1998. CpG is a potent enhancer of specific immunity in mice immunized with recombinant hepatitis B surface antigen. J. Immunol. **160:** 870–876.
6. **Deloran, P., C. Chougnet, J.-P. Lepers, S. Tallet, and P. Coulanges.** 1991. Protective value of elevated levels of γ interferon in serum against exoerythrocytic stages of *P. falciparum.* J. Clin. Microbiol. **29:**1757–1760.
7. **Demi, L., R. Schirmbeck, J. Reimann, H. Wolf, and R. Wagner.** 1999. Immunostimulatory CpG motifs trigger a T helper-1 immune response to human immunodeficiency virus type-1 (HIV) gp160 envelope proteins. Clin. Chem. Lab. Med. **37:**199–204.
8. **DeSouza, J. B., K. H. Williamson, T. Otani, and J. H. Playfair.** 1997. Early gamma interferon responses in lethal and nonlethal murine blood-stage malaria. Infect. Immun. **65:**1593–1598.
9. **Dodoo, D. F. Omer, J. Todd, B. Akanmori, K. Koram, and E. Riley.** 2002. Absolute levels and ratios of pro-inflammatory and anti-inflammatory cytokine production in vitro predict clinical immunity to *P. falciparum* malaria. J. Infect. Dis. **185:**971–979.
10. **Facer, C. A., and M. Tanner.** 1997. Clinical trials of malaria vaccines: progress and prospects. Adv. Parasitol. **39:**1–68.
11. **Favre, N., B. Ryffel, G. Bordmann, and W. Rudin.** 1997. The course of *Plasmodium chabaudi chabaudi* infections in interferon-gamma receptor deficient mice. Parasite Immunol. **19:**375–383.
12. **Gately, M. K., L. M. Renzetti, J. Magram, A. S. Stern, L. Adorini, U. Gubler, and D. H. Presky.** 1998. The interleukin-12/interleukin-12 receptor system: role in normal and pathogenic immune responses. Annu. Rev. Immmunol. **16:**495–521.
13. **Good, M. F.** 2001. Towards a blood-stage vaccine for malaria: are we following all the leads? Nat. Rev. Immunol. **1:**117–125.
14. **Good, M. F., and D. L. Doolan.** 1999. Immune effector mechanisms in malaria. Curr. Opin. Immunol. **11:**412–419.
15. **Gramzinski, R. A., D. L. Doolan, M. Sedegah, H. L. Davis, S. M. Krieg, and S. L. Hoffman.** 2001. Interleukin-12- and gamma interferon-dependent protection against malaria conferred by CpG oligodeoxynucleotide in mice. Infect. Immun. **69:**1643–1649.
16. **Heinzel, F. P., R. M. Rerko, F. Ahmed, and A. M. Hujer.** 1996. IFN-γ independent production of IL-12 during murine endotoxemia. J. Immunol. **157:**4521–4528.
17. **Holder, A. A.** 1999. Malaria vaccines. Proc. Natl. Acad. Sci. USA **96:**1167–1169.
18. **James, S., and L. Miller.** 2000. Malaria vaccine development: status report, p. 9–13. *In* Nature medicine special focus: malaria.
19. **Jankovic, D., P. Caspar, M. Zweig, M. Garcia-Moll, S. D. Showalter, F. R. Vogel, and A. Sher.** 1997. Adsorption to aluminum hydroxide promotes the activity of IL-12 as an adjuvant for antibody as well as type 1 cytokine responses to HIV-1 gp120. J. Immunol. **159:**2409–2417.
20. **Jankovic, D., T. A. Wynn, M. C. Kullberg, S. Hieny, P. Caspar, S. James, A. W. Cheever, and A. Sher.** 1999. Optimal vaccination against *Schistosoma mansoni* requires the induction of both B cell- and IFN-γ-dependent effector mechanisms. J. Immunol. **162:**345–351.
21. **Kenney, R. T., D. L. Sacks, J. P. Sypek, L. Vilela, A. A. Gam, and K. Evans-Davis.** 1999. Protective immunity using recombinant human IL-12 and alum as adjuvants in a primate model of cutaneous leishmaniasis. J. Immunol. **163:**4481–4488.
22. **Kitamura, D., J. Roes, R. Kühn, and K. Rajewsky.** 1991. A B cell-deficient mouse by targeted disruption of the membrane exon on the immunoglobulin mu chain gene. Nature **350:**423–426.
23. **Klinman, D., A.-K. Yi, S. L. Beaucage, J. Conover, and A. M. Krieg.** 1996. CpG motifs present in bacterial DNA rapidly induce lymphocytes to secrete IL-6, IL-12 and IFN-γ. Proc. Natl. Acad. Sci. USA **93:**2879–2883.
24. **Langhorne, J., S. J. Quin, and L. A. Sanni.** 2002. Mouse models of blood-stage malaria infections: immune responses and cytokines involved in protection and pathology, p. 204–228. *In* P. Perlmann and M. Troye-Blomberg (ed.), Malaria immunology. Karger, Basel, Switzerland.
25. **Langhorne, J., B. Simon-Haarhaus, and S. J. Meding.** 1990. The role of $CD4^+$ T cells in the protective immune response to *Plasmodium chabaudi* in vivo. Immunol. Lett. **25:**101–108.
26. **Luty, A. J. F., B. Lell, R. Schmidt-Ott, L. G. Lehman, D. Luckner, B. Greve, P. Matousek, K. Herbich, D. Schmidt, F. Migot-Nabias, P. Deloran, R. S. Nussenzweig, and P. G. Kremnser.** 1999. Interferon-γ responses are associated with resistance to reinfection with *Plasmodium falciparum* in young African children. J. Infect. Dis. **179:**980–988.
27. **Miller, M. A., M. J. Skeen, and H. K. Ziegler.** 1997. A synthetic peptide administered with IL-12 elicits immunity to *Listeria monocytogenes.* J. Immunol. **159:**3675–3679.
28. **Mohan, K., P. Moulin, and M. M. Stevenson.** 1997. Natural killer cell cytokine production, not cytotoxicity, contributes to resistance against blood-stage *Plasmodium chabaudi* AS infection. J. Immunol. **159:**4990–5004.
29. **Mohan, K., and M. M. Stevenson.** 1998. Acquired immunity to asexual blood stages, p. 467–493. *In* I. W. Sherman (ed.), Malaria: parasite biology, pathogenesis, and protection. American Society Microbiology, Washington, D.C.
30. **Near, K. A., A. W. Stowers, D. Jankovic, and D. C. Kaslow.** 2002. Improved immunogenecity and efficacy of the recombinant 19-kilodalton merozoite surface protein 1 by addition of oligodeoxynucleotide and aluminum hydroxide gel in a murine malaria vaccine model. Infect. Immun. **70:**692–701.
31. **O'Garra, A., and K. Murphy.** 1994. Role of cytokines in determining T-lymphocyte function. Curr. Opin. Immunol. **6:**458–466.
32. **Plebanski, M., and A. Hill.** 2000. The immunology of malaria infection. Curr. Opin. Immunol. **12:**437–441.
33. **Podoba, J. E., and M. M. Stevenson.** 1991. $CD4^+$ and $CD8^+$ T lymphocytes both contribute to acquired immunity to blood-stage *Plasmodium chabaudi* AS. Infect. Immun. **59:**51–58.
34. **Sam, H., and M. M. Stevenson.** 1999. In vivo IL-12 production and IL-12

receptors β1 and β2 mRNA expression in the spleen are differentially upregulated in resistant B6 and susceptible A/J mice during early blood-stage *Plasmodium chabaudi* AS malaria. J. Immunol. **162:**1582–1589.
35. **Stacey, K. J., and J. M. Blackwell.** 1999. Immunostimulatory DNA as an adjuvant in vaccination against *Leishmania major.* Infect. Immun. **67:**3719–3726.
36. **Stevenson, M. M., J. J. Lyanga, and E. Skamene.** 1982. Murine malaria: genetic control of resistance to *Plasmodium chabaudi.* Infect. Immun. **38:**80–88.
37. **Stevenson, M. M., M. F. Tam, S. F. Wolf, and A. Sher.** 1995. IL-12 induced protection against blood-stage *Plasmodium chabaudi* AS requires IFN-γ and TNF-α and occurs via an NO-dependent mechanism. J. Immunol. **155:**2545–2556.
38. **Stevenson, M. M., and M. F. Tam.** 1993. Differential induction of helper T cell subsets during blood-stage *Plasmodium chabaudi* AS infection in resistant and susceptible mice. Clin. Exp. Immunol. **92:**77–83.
39. **Su, Z., and M. M. Stevenson.** 2002. IL-12 is required for antibody-mediated protective immunity against blood-stage *Plasmodium chabaudi* AS malaria infection in mice. J. Immunol. **168:**1348–1355.
40. **Su, Z., and M. M. Stevenson.** 2000. Central role of endogenous gamma interferon in protective immunity against blood-stage *Plasmodium chabaudi* AS infection. Infect. Immun. **68:**4399–4406.
41. **Taylor-Robinson, A. W., and R. S. Philips.** 1994. B cells are required for the switch from TH1- to TH2-regulated immune response to *Plasmodium chabaudi chabaudi* infection. Infect. Immun. **62:**2490–2498.
42. **van der Heyde, H. C., B. Pepper, J. Batchelder, F. Cigel, and W. P. Weidanz.** 1997. The time course of selected malarial infections in cytokine-deficient mice. Exp. Parasitol. **88:**206–213.
43. **von der Weid, T., and J. Langhorne.** 1993. Altered response of $CD4^+$ T cell subsets to *Plasmodium chabaudi chabaudi* in B cell-deficient mice. Int. Immunol. **5:**1343–1348.
44. **von der Weid, T., N. Honarvar, and J. Langhorne.** 1996. Gene-targeted mice lacking B cells are unable to eliminate a blood stage malaria infection. J. Immunol. **156:**2510–2516.
45. **Weeratna, R. D., M. J. McCluskie, Y. Xu, and H. S. Davis.** 2000. CpG DNA induces stronger immune responses with less toxicity than other adjuvants. Vaccine **18:**1755–1762.
46. **Wynn, T. A., A. W. Cheever, D. Jankovic, R. W. Poindexter, P. Caspar, F. A. Lewis, and A. Sher.** 1995. An IL-12-based vaccination method for preventing fibrosis induced by schistosome infection. Nature **376:**594–596.
47. **Wynn, T. A., D. Jankovic, S. Hieny, A. W. Cheever, and A. Sher.** 1995. IL-12 enhances vaccine-induced immunity to *Schistosoma mansoni* in mice and decreases T helper 2 cytokine expression, IgE production, and tissue eosinophilia. J. Immunol. **154:**4701–4709.
48. **Wynn, T. A., A. Reynolds, S. James, A. W. Cheever, P. Caspar, S. Hieny, D. Jankovic, M. Strand, and A. Sher.** 1996. IL-12 enhances vaccine-induced immunity to schistosomes by augmenting both humoral and cell-mediated immune responses against the parasite. J. Immunol. **157:**4068–4078.
49. **Yap, G. S., and M. M. Stevenson.** 1994. Differential requirements for an intact spleen in induction and expression of B-cell-dependent immunity to *Plasmodium chabaudi* AS. Infect. Immun. **62:**4219–4225.

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Mitogenic synthetic polynucleotides suppress the antibody response to a bacterial polysaccharide

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*Unmethylated bacterial DNA containing a high frequency of the CpG motif, is mitogenic and induces T-cell independent, murine B-cell proliferation. These stimulatory effects are also induced by synthetic oligonucleotides that contain one or more unmethylated CpG dinucleotides (CpG oligo). Such mitogenicity is not seen with highly methylated vertebrate DNA, which has a lower prevalence of the CpG motif than bacterial DNA. Due to their stimulatory effects, CpG oligo have been proposed for use as vaccine adjuvants. In order to determine if a synthetic CpG oligo that was stimulatory for B-cell proliferation could augment the murine antibody response to protective bacterial polysaccharide epitopes (*Pseudomonas aeruginosa *LPS-O polysaccharide side chain; high-molecular-weight polysaccharide or high-MW PS), BALB/c mice were injected with mitogenic doses of CpG oligo simultaneously with high-MW PS, and antibody titers were measured by ELISA weekly for 4 weeks. Controls received PBS, a nonstimulatory control oligo plus PS, CpG alone, or PS alone. Despite evidence of B-cell mitogenicity and an increase in total IgM in CpG oligo-treated mice, CpG oligo treatment plus PS significantly decreased the high-MW PS antibody response compared to PS alone. The blunting of the anti-PS antibody response could be eliminated by vaccinating the animals with PS prior to CpG oligo. We conclude that despite in vitro and in vivo evidence of B-cell proliferation, this CpG oligo reduces PS-specific antibody responses in an animal model when given simultaneously with a bacterial polysaccharide. Based on results in this model, oligonucleotides containing stimulatory unmethylated CpG dinucleotides may not be useful adjuvants when given simultaneously with bacterial PS vaccines. © 1997 Elsevier Science Ltd.*

Pseudomonas aeruginosa continues to be a significant human pathogen despite the availability of appropriate antimicrobial agents. Specific groups of individuals such as neutropenic cancer patients, individuals with burns, and children with cystic fibrosis are particularly susceptible to serious infections with *Pseudomonas aeruginosa*[1,2]. Antibodies directed to certain surface polysaccharides (PS) of *Pseudomonas*, in conjunction with phagocytes, are often protective against infection with this organism. Antibodies against lipopolysaccharide *O*-specific side chain (high-MW PS) for example, supply serotype-specific protection[3].

The immune response to bacterial polysaccharides differs from immune responses to protein antigens. Polysaccharide antigens are 'T-independent' in character, do not commonly elicit booster responses with repeat immunization, and elicit antibodies of restricted clonotype and isotype (IgM and IgG3 in mice, IgM, IgG2 and IgG1 in humans)[4,5]. In addition, polysaccharide antigens are particularly poor immunogens in small children under the age of two years and in immunocompromised adults. Conjugation of bacterial polysaccharides to proteins results in immunogens that act as partially 'T-dependent' antigens, and have greatly improved immunogenicity for some bacterial polysaccharides such as the capsule of *Haemophilus influenzae* type b[6,7]. Current polysaccharide–protein conjugate vaccines, however, require multiple doses to achieve protective antibody levels in children. In addition, some polysaccharides from bacterial pathogens are poor immunogens even when conjugated to appropriate carrier proteins[8,9].

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Thus adjuvants that could enhance the anti-polysaccharide antibody response could have important clinical benefit.

Bacterial DNA has recently been proposed as a possible adjuvant for polysaccharide antigens and other poorly immunogenic compounds. Bacterial DNA has been shown to be a potent activator for murine splenocytes, including B cells, NK cells, and $CD4^+$ T cells in both in vitro and in vivo experiments[10–20]. By contrast, vertebrate DNA treatment is not stimulatory in these models. Oligonucleotides containing nonmethylated CpG motifs not normally present in vertebrate DNA but frequently occurring in bacterial DNA, have also been shown to activate lymphoid cells[10–13,15,17,21–23]. In particular, when purified splenic B cells were cultured with CpG, nonCpG or nonstimulatory CpG oligos (NSCpG), tritiated thymidine and tritiated uridine incorporation was dramatically increased only in CpG-treated B-cells[13,15,17]. This evidence of in vitro activation was further demonstrated by data showing an increase in IgM secretion by CpG-treated B lymphocytes[13,20]. Finally, short-term in vivo experiments involving intraperitoneal injection of mice with phosphorothioate CpG, nonCpG or NSCpG oligos followed 24 h later by splenectomy, demonstrated that CpG-treated B cells had been induced in vivo to proliferate[13]. As a result of these in vitro and other short-term in vivo results, some investigators have suggested that oligonucleotides containing nonmethylated stimulatory CpG motifs could be used as adjuvants for inducing an improved immune response to normally poor immunogens[13]. The current study was performed to determine if the simultaneous administration of stimulatory CpG oligo and high-MW PS could increase the anti-PS antibody response in an animal model.

MATERIALS AND METHODS

Bacterial polysaccharide

The high molecular weight polysaccharide (high-MW PS) component of Fisher–Devlin immunotype I *Pseudomonas aeruginosa* lipopolysaccharide *O*-specific side chain was isolated from bacterial culture supernatants as previously described[24]. This material contains the immunodeterminants of the *O*-specific side chain but does not contain lipid A or nucleic acids; it was kindly provided by Dr. Gerald Pier, Channing Laboratories, Harvard Medical School, Boston.

Phosphorothioate DNA oligonucleotides

Unmethylated oligonucleotides including either a stimulatory CpG motif (CpG) or control nonstimulatory CpG motif (NSCpG) were purchased from Operon (Almeda, CA). The two oligonucleotide sequences were chosen on the basis of previous reports of their immunostimulatory properties and were:

(1) stimulatory CpG oligo '1d' 5′ GCATGACGTTGAGCT 3′ so that the CpG motif is flanked by two 5′ purines and two 3′ pyrimidines;
(2) nonstimulatory CpG oligo '1a': 5′ GCTAGATGTTAGCGT 3′ containing a CpG motif but flanked by two 5′ purines and only one 3′ pyrimidine.

These sequences have been previously demonstrated to be stimulatory or nonstimulatory, respectively, to mammalian B cells[13,20].

Animals

8–14-week-old female BALB/c ByJ pathogen-free mice (Jackson Laboratories, Bar Harbor, Maine) were maintained in a *Pseudomonas*-free animal facility. Drinking water was filtered and autoclaved, and bedding, food and microisolator cages were autoclaved before use. We have previously shown that animals remaining in this facility do not become pseudomonas-colonized and do not seroconvert[3,25].

Long-term animal experiments (duration 1 month)

The BALB/cByJ mice were bled via tail-vein puncture and then injected intraperitoneally with either phosphate-buffered saline (PBS), 10 μg high-MW PS, 500 μg NSCpG or CpG oligo or a combination of oligonucleotide plus polysaccharide (either 250 μg or 500 μg oligo plus 10 μg high-MW PS). Mice were bled once weekly for 4 weeks, and sera were analyzed for anti-high-MW PS antibodies by ELISA, as described below (Section 1.5)[3].

Enzyme-linked immunosorbent assay (ELISA) to detect anti-high-MW PS antibodies

ELISA was used to detect anti-high-MW PS antibodies in mouse sera as we have previously described[3,25]. High-MW PS was tyraminated via cyanogen bromide coupling of tyramine to the PS in order to enhance binding to the ELISA plate[3,24,25]. Briefly, tyraminated PS was coated on 96-well microtiter plates (Immunlon 4, Dynatech) at a concentration of 1 μg ml^{-1} (100 μl well^{-1}) overnight at 4°C. Plates were washed with PBS–Tween and blocked with 1% BSA in PBS. After three additional PBS–Tween washes, mouse sera (diluted 1:100) were loaded (100 μl well^{-1}) and incubated overnight at 4°C. Goat anti-mouse Ig (H+L) alkaline phosphatase conjugate (AP; Fisher/Southern Biotech, Atlanta, GA) was diluted 1:1000 and added to washed plates. After excess conjugate was washed off, plates were developed with diethanolamine (DEA) buffer and *p*-nitrophenol phosphate (pNPP, Sigma, St. Louis, MO) using standard procedures, and then read on an ELISA plate reader (Dynatech) at 410 nm. Normalization of data from each plate was performed using a standard curve based on binding of a murine monoclonal antibody specific for high-MW PS that we have previously described[3]. The average slope of all the monoclonal antibody standard curves for an experiment was calculated and then used to normalize the data, thus minimizing plate-to-plate variation. Once raw data were normalized, the mean OD per treatment group for each time point was calculated. Standard error of the mean (SEM) was used to generate error bars for graphical comparisons of the mean ODs. The seroconversion frequency defined as the number of animals having OD measurements of threefold or greater than the background of the PS-specific ELISA divided by the total number of animals in that treatment group, was also determined. χ^2 analysis was utilized to compare the seroconversion frequencies of the oligo treatment groups to the PS alone treatment group.

ELISA to measure total serum IgM

ELISA was used to measure total IgM levels in mouse sera. Briefly, goat anti-mouse Ig (H+L; Fisher/Southern Biotech) was coated on microtiter plates (Corning) at a concentration of 5 μg ml^{-1} (100 μl well^{-1}) overnight at 4°C. Washed plates were blocked with 1% BSA, washed three times and loaded with mouse serum samples (dilution 1:10000, 100 μl well^{-1}) for overnight incubation at 4°C. After three washes, goat anti-mouse IgM AP (Fisher/Southern Biotech) diluted 1:1000 was added to the plates. Plates were developed with DEA buffer and pNPP using standard procedures, and read on a Dynatech ELISA plate reader at 410 nm. Conversion of optical density (OD) measurements to mg ml^{-1} IgM was based on IgM standard curves generated for each ELISA plate with a purified murine IgM standard (Fisher/Southern Biotech). Mean IgM levels $\pm$ standard error of the mean (SEM) were calculated for each treatment group at each time point.

Short-term animal experiments (duration 1 day)

BALB/c mice were injected intraperitoneally with the same reagents used for the long-term experiments, but were sacrificed after 24 h. Splenocytes were isolated from spleens and stained for flow-cytometric analysis as described below (Section 1.8).

Two-color flow cytometric analysis of splenocytes from immunized mice

Spleens from mice receiving various immunizations were pressed between frosted microscope slides to release the splenocytes. Debris was allowed to settle and then the supernatant was collected and centrifuged. Red cells were lysed prior to antibody staining according to standard procedures[26,27]. Antibodies selected for staining of splenocytes included fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgM (Fisher/Southern Biotech), FITC-labeled mouse anti-mouse Ia_d (PharMingen, San Diego, CA), FITC-labeled hamster anti-mouse CD3ε (PharMingen) and phycoerythrin (PE)-labeled rat anti-mouse CD45R (B220; PharMingen). Control staining was also performed with FITC- or PE-labeled isotype-matched antibodies. Additionally, rat anti-mouse CD32/CD16 (Fc block™, PharMingen) was used prior to incubation of splenocytes with the staining reagents to decrease background staining. Antibodies were used so that 50 μl of the Fc blocking antibody at 20 μg ml^{-1} was added to 50 μl of splenocytes at 2×10^7cells ml^{-1} for 5 min on ice, followed by addition of the same concentration of staining antibodies for 30 min on ice. Cells were double-stained with anti-B220 and either anti-IgM, anti-Ia^d or anti-CD3ε as appropriate. After two washes, cells were analyzed on a Becton Dickinson FACScan flow cytometer.

RESULTS

Detection of antibodies to LPS *O*-side chain polysaccharide in CpG oligo and polysaccharide-immunized mice

The adjuvant activity of CpG oligonucleotide was assessed by immunizing mice with a bacterial polysaccharide (high-MW PS) in combination with CpG as well as NSCpG oligonucleotides. Antigen-specific ELISA was then performed on sera from immunized mice. Simultaneous immunization of mice with CpG oligo and *Pseudomonas aeruginosa* LPS *O*-side chain polysaccharide (high-MW PS) yielded lower antibody titers than control animals given nonCpG oligo plus polysaccharide or polysaccharide alone (*Figure 1*A and B). NSCpG oligo plus PS-immunized mice also exhibited slightly decreased antibody titers (*Figure 1*A and B), however, these titers were substantially higher than the CpG oligo plus PS-immunized mice. In addition to decreased antibody titers, CpG oligo plus PS-immunized mice seroconverted (defined as an increase in ELISA OD to three times the background) to polysaccharide significantly less frequently than animals immunized with PS and NSCpG oligo or with PS alone (*Table 1*). Thus, use of CpG oligo simultaneously with an immunogenic bacterial PS resulted in a decreased titer of PS-specific antibody and in a reduction in frequency of seroconversion. These results were unexpected because of the mitogenic activity of CpG oligonucleotides for mouse B cells.

Measurement of serum IgM in immunized mice

Total serum IgM levels were measured to determine if CpG oligo was appropriately stimulatory to B cells in

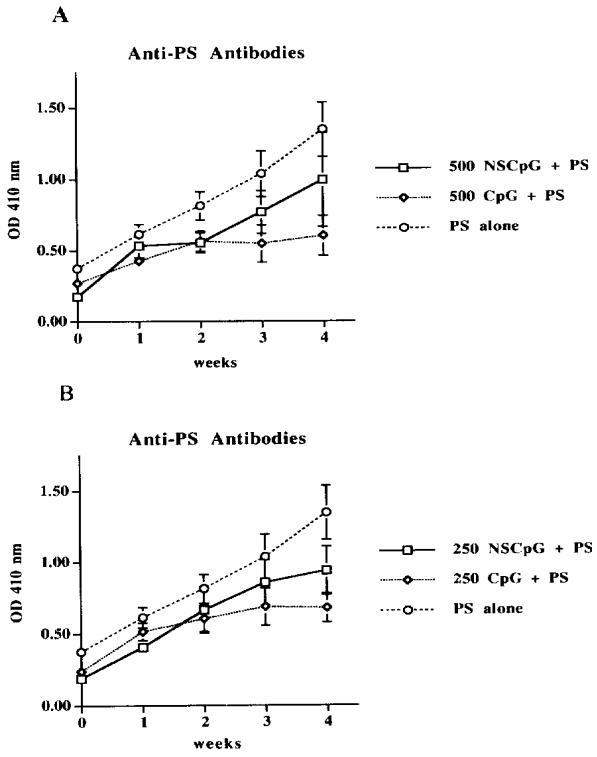

Figure 1 The effect of CpG oligo administration on seroconversion of mice to *Pseudomonas aeruginosa* high-MW PS. An antigen-specific ELISA described in Section 1.5 was used to evaluate sera for antibodies to PS. Mice were immunized with 10 μg PS either alone or in combination with 500 μg NSCpG or CpG oligo (A) or in combination with 250 μg NSCpG or CpG oligo (B). Each treatment group contained five mice, and points represents means from three different experiments

Table 1 CpG oligo administration decreases the percentage of animals seroconverting to *Pseudomonas aeruginosa* high-molecular-weight polysaccharide

Treatment	Number of mice (% seroconversion)
PBS	0/10 (0)
PS	15/15 (100)
500CpG+PS	7/15 (53)[a]
250CpG+PS	3/10 (30)[b]
500NSCpG+PS	13/16 (81)
250NSCpG+PS	9/10 (90)
PS/PBS[c]	3/3 (100)
PS/250CpG[c]	3/3 (100)
PS/250NSCpG[c]	3/3 (100)

%Seroconversion = (# of animals whose OD at 410 nm reached 3× the background for the ELISA/# of animals receiving the treatment) ×100. Data were compared by χ^2 analysis to determine significance.
[a]$P < 0.0025$ versus PS.
[b]$P < 0.0001$ versus PS.
[c]Animals were immunized with polysaccharide followed 48 h later by PBS, CpG or non-CpG oligos.

vivo in our experimental system as previously described[13]. Total IgM levels were increased in CpG oligo-immunized animals as compared to animals given PBS or NSCpG oligo, consistent with B-cell mitogenicity of the CpG oligo in vivo (*Figure 2*A). In addition, animals simultaneously treated with 500 μg CpG oligo plus PS had elevated total IgM levels compared to animals immunized simultaneously with 500 μg NSCpG oligo plus PS (*Figure 2*B). Thus, the increased total IgM levels in mice treated simultaneously with CpG oligo and bacterial PS suggests that failure to upregulate B cells or interference of PS with CpG oligo mitogenicity was not the explanation for the depressed PS-specific antibody levels.

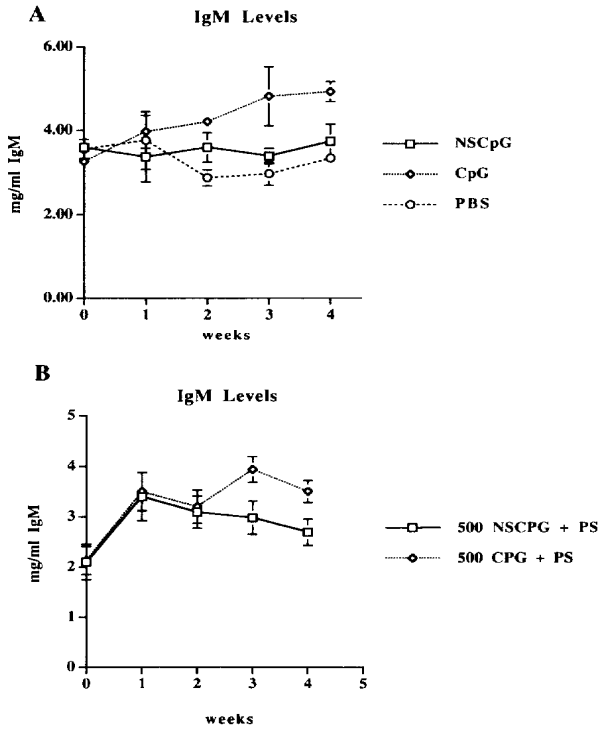

Figure 2 (A) The effect of CpG oligo administration on the total serum IgM levels in mice. Mice were immunized with PBS, NSCpG oligo or CpG oligo (500-μg dose only). An isotype-specific ELISA was used to evaluate mouse sera for total IgM (Section 1.6). The IgM levels were higher in mice immunized with 500 μg CpG oligo compared to mice immunized with NSCpG or PS alone. (B) Total serum IgM of mice immunized with CpG oligo simultaneously with PS was also increased compared to NSCpG plus PS controls. The data represent means from three experiments with a total of 15 mice per treatment group

Flow cytometry analysis of BALB/c mouse splenocytes after in vivo exposure to either nonCpG or CpG oligo

Because experiments over four weeks showed a suppressive effect of CpG oligo on polysaccharide-specific antibody levels, flow cytometry experiments were performed in order to determine whether the CpG oligo was acting appropriately as a B-cell mitogen in our experimental systems. Short-term animal experiments (i.e. splenocytes collected 24 h after vaccination with oligo or PBS) confirmed that the CpG oligo was a B-cell mitogen in our experiments. The number of B cells in CpG-treated mice was increased as assessed by IgM and Ia^d expression (*Figure 3*, *Table 2*). Additionally, the mean fluorescence measurement of Ia^d was much higher in CpG-treated mice, indicating an increase in the surface expression of this marker (*Table 2*). Splenocytes from animals simultaneously treated with PS and CpG oligo showed a similar pattern to that seen with CpG oligo treatment alone, indicating that the PS was not affecting the ability of the CpG oligo to perform as a B-cell mitogen in vivo (*Table 2*).

Polysaccharide pretreatment to assess the importance of timing of oligonucleotide vaccination to the anti-polysaccharide antibody response

To investigate whether the antigen-specific suppression seen in four-week-long animal experiments was the consequence of the simultaneous administration of the polysaccharide antigen with the polyclonal stimulator (i.e. CpG oligo), animals were first immunized with PS, and then vaccinated with CpG oligo 48 h later. Animals immunized first with PS followed 48 h later by CpG oligo were more likely to seroconvert to PS than those simultaneously immunized with CpG oligo and PS (*Figure 4*, *Table 1*). In addition, more animals in the groups receiving PS first followed by CpG oligo seroconverted to PS (100%) compared to mice simultaneously immunized with PS and 500 μg CpG or 250 μg CpG (53% and 30% seroconversion respectively). Finally, over the four-week experiment, the mean high-MW PS antibody titers in the animals first immunized with PS followed later by CpG oligo was higher than those seen in mice receiving PS and CpG simultaneously (*Figure 1*A and B and *Figure 4*).

DISCUSSION

Synthetic oligonucleotides that are CpG-rich and unmethylated, thus mimicking bacterial DNA, have been suggested as adjuvants and biological response modifiers due to their immunostimulatory properties[13]. Previous studies with bacterial DNA or CpG oligos focused primarily on their ability to stimulate in vitro cultures of murine splenocytes and human lymphocytes[10–23]. More recently, some investigators have shown

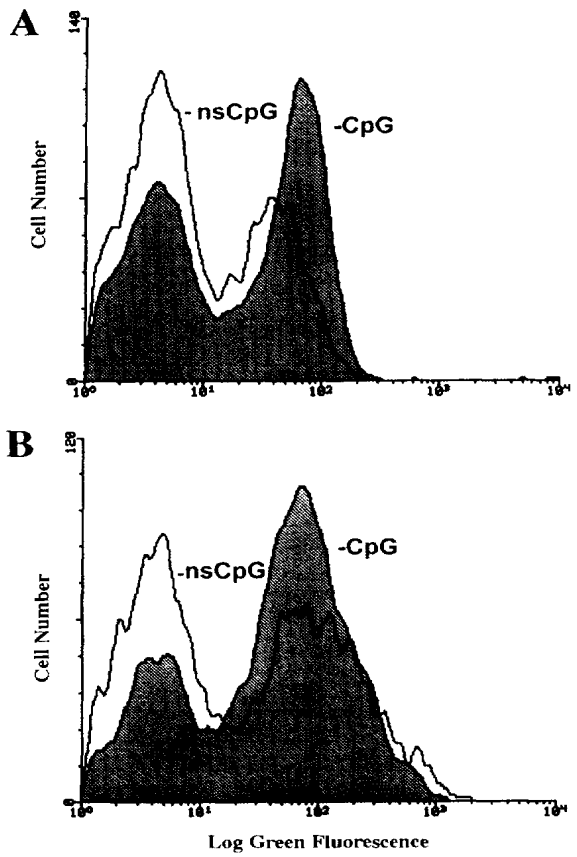

Figure 3 Flow cytometry analysis demonstrating the mitogenic effect of CpG immunization on mouse splenic B cells. Total B cell number was increased in the CpG oligo-immunized mice but not in control mice given NSCpG oligos. Panel A shows the staining of mouse splenocytes from immunized animals by FITC-labelled mouse anti-mouse class II (Ia^d). Panel B shows the staining of mouse splenocytes by FITC labelled goat anti-mouse IgM. For both panels, shaded histograms depict staining for CpG oligo-immunized mice, and open histograms staining for NSCpG oligo recipients. The experiment was repeated three times with similar results. PS addition to the oligo injection mixture did not affect the mitogenic properties of the oligos, and PS alone was not mitogenic in the assay (not shown)

Table 2 Effects of CpG oligo and PS immunization on proliferation and class II MHC expression of mouse splenic B cells

Treatment	B cells/T cells	Mean channel fluorescence Ia^d
PBS	0.517	36.39
500NSCpG	0.560	38.53
500CpG	0.786	46.37
PS	0.544	32.96
250NSCpG+PS	0.501	32.49
250CpG+PS	0.718	41.37

The B- to T-cell ratios were calculated based on the number of cells staining with α-B220 (B cells) and the number of cells staining with α-Cd3ε (T cells). The ratios were not different for cells stained with α-IgM and a-Ia^d. The results indicated are from one animal per treatment group. With the exception of the PS-alone treatment group, two or more animals were evaluated for each treatment.

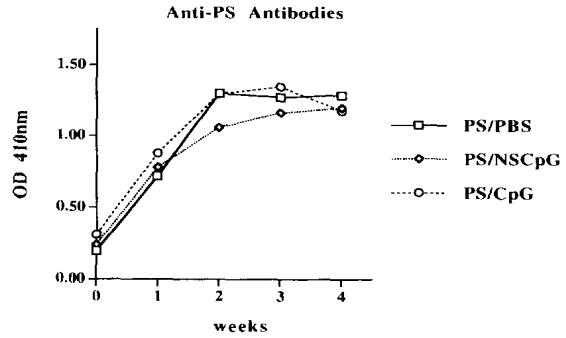

Figure 4 Immunization of mice with high-MW PS prior to CpG oligo restores the PS-specific antibody response. Mice were administered 10 μg PS followed 48 h later by administration of PBS, NSCpG or CpG oligo (250-μg dose). The PS-specific ELISA was then used to evaluate sera for antibody titer to PS. Each treatment group contained three mice

synthetic oligos are B-cell mitogens in vivo and yield increased production of a variety of cytokines as well as IgM[10,13,20]. These experiments only examined short-term (1–3 days) immune responses in mice and did not investigate the effect of CpG oligos on pathogen-specific immunity. In the present study, we focused on a longer term (1 month) in vivo mouse model for the antigen specific seroconversion to the high-MW PS component of Fisher–Devlin immunotype I *Pseudomonas aeruginosa* lipopolysaccharide *O*-side chain[3]. In this model, adult BALB/c mice seroconvert to high-MW PS after one immunization, and the antibodies persist for more than 1 month. We hypothesized that immunization of mice with CpG oligo simultaneously with PS would elevate the levels of PS-specific antibody produced as compared to mice immunized with PS alone or with the nonstimulatory NCpG oligo plus PS, since CpG oligo was an effective mitogen for B cells. In contrast to this expectation, polysaccharide-specific antibody levels were reduced in the mice coadministered CpG oligo and high-MW PS, as compared to mice administered high-MW PS with NSCpG oligo or PS alone without 'adjuvant'. Two different doses of CpG oligo were suppressive in terms of the number of animals seroconverting to PS and in the titer of anti-PS antibody produced.

Thus, results from in vivo experiments in which mice were immunized simultaneously with high-MW PS from *Pseudomonas aeruginosa* and CpG oligo yielded unexpected results over the 4-week duration of the experiment. Rather than finding CpG oligo to be an effective adjuvant for a bacterial polysaccharide antigen, we found suppression of PS-specific antibody levels, and a significantly decreased frequency of seroconversion. Since bacterial infection in humans exposes the host to bacterial surface PS and bacterial DNA, it seems possible that the nonspecific mitogenicity of bacterial DNA is not optimal for an immune response to surface polysaccharides and may be a bacterial adaptation to evade host defenses. Alternatively, the polyclonal stimulation of mammalian lymphocytes in response to bacterial DNA or nonmethylated CpG oligos may be unrelated to host–pathogen interactions, but rather may result from differences in evolutionary pathways in which vertebrates, unlike prokaryotes, followed a path to CpG

motif suppression in their DNA. Further experiments will be needed to define fully the mechanism of CpG-mediated LPS *O*-side chain antibody suppression, and to determine if the reduced titers of anti-PS antibodies are seen when other bacterial PS are used as vaccines with CpG oligos. Interestingly, NSCpG oligo immunization with PS resulted in a slight decrease in serum anti-PS antibody titer as well as a nonsignificant decrease in the frequency of mouse seroconversion, suggesting that this DNA oligo may have some immunomodulatory effect. This oligo was chosen because it was nonstimulatory in previous experiments. However, it does contain a CpG motif that is not flanked by two 3′ pyrimidines needed for optimal immunostimulatory effect[13]. It is therefore possible that some immunomodulation may have occurred utilizing this control oligo not detected in our in vitro experiments. Additional immunization experiments in animals utilizing stimulatory CpG, nonstimulatory CpG as well as nonCpG oligos should help provide the information necessary to fully evaluate the potential of stimulatory CpG oligos as adjuvants for immunization with bacterial PS.

Once polysaccharide-specific antibody levels were determined to be suppressed in PS/CpG-immunized mice compared to PS-alone and PS/NSCpG-immunized mice, total IgM levels were evaluated. Total serum IgM levels in CpG-immunized mice (both with and without PS) were elevated in comparison to NSCpG immunized (also with and without PS) mice. These results are consistent with CpG oligos being mitogenic in these in vivo studies and suggest that the reduction in PS-specific antibody titers was not due to global immune suppression.

Short-term animal experiments, which measured the number of splenic B cells 24 h after immunization of mice with CpG and NSCpG oligos confirmed previous results that CpG oligos are B-cell mitogens. The coadministration of PS and CpG oligo in these experiments did not result in changes in mitogenicity of the CpG oligo. Additionally, the PS alone was not mitogenic which is consistent with the previously reported low level of nucleic acid and LPS contamination of the high-MW PS preparations and previous data with purified carbohydrate antigens[3,5,24].

The broad polyclonal immunostimulatory effects of bacterial DNA and CpG oligos may not be ideal for simultaneous production of anti-bacterial PS antibodies. CpG oligos have been found to increase production of cytokines such as IL-6, a cytokine response that may not be optimal for antibody responses to T-cell independent type 2 bacterial polysaccharide antigens[20]. IL-6 is known to be a potent inducer of B-cell differentiation and may unfavorably alter B-cell interactions with polysaccharide antigens. Alternatively, increased surface IgM as a result of B-cell exposure to CpG oligo may increase receptor occupancy by the PS on the surface of the B cell and induce tolerance. Overly restrictive crosslinking of B-cell surface receptors has recently been shown to induce tolerance in hapten-specific B cells[27]. It is also possible that timing of the use of CpG oligo may be important so that use of oligo after PS immunization might stimulate B cells already committed to PS-specific antibody production and thus be an effective adjuvant. Our data showing recovery of the PS-specific antibody titer if CpG oligo are utilized after immunization with PS support this premise.

Thus, further experiments will be needed to define fully the mechanism of CpG-mediated LPS *O*-side chain antibody suppression, to determine whether suppression is seen when other bacterial PS or nonPS antigens are used with the CpG oligos, and to determine if different timing of the use of CpG oligo immunization can enhance the anti-PS antibody response. It will also be important to determine if IL-6 or other CpG-induced mediators cause an increase or decrease in B-cell responsiveness to PS antigens. Additional immunization experiments in animals should provide the information necessary to evaluate more fully the potential of CpG oligos as vaccine adjuvants.

REFERENCES

1 Rabin, E.R., Graber, E.G. Vogal, Finkelstein, R.A. and Tumbusch, W.A. Fatal pseudomonas infection in burned patients. A clinical, bacteriologic and anatomic study. *N. Engl. J. Med.* 1961, **265,** 1231–1255.

2 Schreiber, J.R. and Goldmann, D., Infections complicating cystic fibrosis. In *Current Clinical Topics in Infectious Diseases*, ed. J.S. Remington and M. Schwartz, McGraw-Hill, New York. 1986, pp. 51-81.

3 Schreiber, J.R., Patawaran, M., Tosi, M.F., Lennon, J. and Pier, G.B. Anti-idiotype-induced, lipopolysaccharide-specific antibody response to *Pseudomonas aeruginosa*. *J. Immunol.* 1990, **144,** 1023–1029.

4 Perlmutter, R.M., Hansberg, D., Briles, D.E., Nicoletti, R.A. and Davie, J.M. Subclass restriction of murine anti-carbohydrate antibodies. *J. Immunol.* 1978, **121,** 566–572.

5 Stein, J.E. Thymus-independent and thymus-dependent responses to polysaccharide antigens. *J. Infect. Dis.* 1992, **165,** 549–552.

6 Robbins, J.B. and Schneerson, R. Polysaccharide–protein conjugates: a new generation of vaccines. *J. infect. Dis.* 1990, **161,** 821–832.

7 Siber, G.R. Pneumococcal disease: prospects for a new generation of vaccines. *Science* 1994, **265,** 1385–1387.

8 Paoletti, L.C., Kasper, D.L., Michon, F., DiFabio, J., Jennings, H.J., Tosteson, T.D. and Wessels, M.R. Effects of chain length on the immunogenicity in rabbits of tetanus toxoid conjugates. *J. Clin. Invest.* 1992, **89,** 203–209.

9 Stein, J.E. Glycoconjugate vaccines: What next? *Technol. Assess. Health Care* 1994, **10,** 167–176.

10 Cowdery, J.S., Chace, J.H., Yi, A.-K. and Krieg, A.M. Bacterial DNA induces NK cells to produce IFN-ϒ in vivo and increases the toxicity of lipopolysaccharides. *J. Immunol.* 1996, **156,** 4570–4575.

11 Halpern, M.D., Kurlander, R.J. and Posetsky, D.S. Bacterial DNA induces murine interferon-ϒ production by stimulation of interleukin-12 and tumor necrosis factor-α. *Cell. Immunol.* 1996, **167,** 72–78.

12 Klinman, D.M., Yi, A.-K., Beaucage, S.L., Conover, J. and Krieg, A.M. CpG motifs present in bacterial DNA rapidly induce lymphocytes to secrete interleukin 6, interleukin 12, and interferon ϒ. *Proc. Natl. Acad. Sci. USA* 1996, **374,** 546–549.

13 Krieg, A.M., Yi, A.-K., Matson, S., Waldschmidt, T.J., Bishop, G.A., Teasdale, R., Koretzky, G.A. and Klinman, D.M. CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature* 1995, **374,** 546–549.

14 Messina, J.P., Gilkeson, G.S. and Pisetsky, D.S. Stimulation of in vitro murine lymphocyte proliferation by bacterial DNA. *J. Immunol.* 1991, **147,** 1759–1764.

15 Messina, J.P., Gilkeson, G.S. and Pisetsky, D.S. The influence of DNA structure on the in vitro stimulation of murine lymphocytes by natural and synthetic polynucleotide antigens. *Cell. Immunol.* 1993, **147,** 148–157.

16 Pisetsky, D.S. The immunologic properties of DNA. *J. Immunol.* 1996, **156,** 421–423.

17 Pisetsky, D.S. and Reich, C. Stimulation of in vitro proliferation of murine lymphocytes by synthetic oligodeoxynucleotides. *Mol. Biol. Rep.* 1993, **18,** 217–221.
18 Yamamoto, S., Kuramoto, E., Shimada, S. and Tokunaga, T. In vitro augmentation of natural killer cell activity and production of interferon-α/β and -γ with deoxyribonucleic acid fraction from *Mycobacterium bovis* BCG. *Jpn. J. Cancer Res.* 1988, **79,** 866–873.
19 Yamamoto, S., Yamamoto, S., Shimada, S., Kuramoto, E., Yano, O., Kataoka, T. and Tokunaga, T. DNA from bacteria, but not from vertebrates, induces interferons, activates natural killer cells and inhibits tumor growth. *Microbiol. Immunol.* 1992, **36,** 983–997.
20 Yi, A.-K., Chace, J.H., Cowdery, J.S. and Krieg, A.M. IFN-γ promotes IL-6 and IgM secretion in response to CpG motifs in bacterial DNA and oligodeoxynucleotides. *J. Immunol.* 1996, **156,** 558–564.
21 Yamamoto, S., Yamamoto, T., Kataoka, T., Kuramoto, E., Yano, O. and Tokunaga, T. Unique palindromic sequences in synthetic oligonucleotides are required to induce INF and augment INF-mediated natural killer activity. *J. Immunol.* 1992, **148,** 4072–4076.
22 Yamamoto, T., Yamamoto, S., Kataoka, T., Komuro, K., Kohase, M. and Tokunaga, T. Synthetic oligonucleotides with certain palindromes stimulate interferon production of human peripheral blood lymphocytes in vitro. *Jpn. J. Cancer Res.* 1994, **85,** 775–779.
23 Yamamoto, T., Yamamoto, S., Kataoka, T. and Tokunaga, T. Lipofection of synthetic oligodeoxyribonucleotide having a palindromic sequence of AACGTT to murine splenocytes enhances interferon production and natural killer activity. *Microbiol. Immunol.* 1994, **38,** 831–836.
24 Pier, G.B., Sidberry, H.F., Zolyomi, S. and Sadoff, J.C. Isolation and characterization of a high molecular weight polysaccharide from the slime of *Pseudomonas aeruginosa*. *Infect. Immun.* 1978, **22,** 908–918.
25 Schreiber, J.R., Nixon, K.L., Tosi, M.F., Pier, G.B. and Patawaran, M.B. Anti-idiotype induced, lipopolysaccharide-specific antibody response to *Pseudomonas aeruginosa*, II. Isotype and functional activity of the anti-idiotype-induced antibodies. *J. Immunol.* 1991, **146,** 188–193.
26 Kruisbeek, A.M., Isolation and fractionation of mononuclear cell populations. In *Current Protocols in Immunology*, ed. J.E. Coligan et al., Greene Publishing and Wiley–Inter Science, New York, 1993, pp. 3.1.1–3.1.5.
27 Mond, J.J. and Brunswick, M., Early parameters of B cell activation. *Current Protocols in Immunology*, ed. J.E. Coligan et al., Greene Publishing and Wiley–Inter Science, New York, 1991, 3.9.1.-3.9.7.

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CpG oligonucleotides improve the protective immune response induced by the anthrax vaccination of rhesus macaques

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Abstract

Synthetic oligodeoxynucleotides (ODN) containing unmethylated CpG motifs act as immune adjuvants, improving the immune response elicited by co-administered vaccines. Combining CpG ODN with anthrax vaccine adsorbed (AVA, the licensed human vaccine) increased the speed, magnitude and avidity of the resultant anti-anthrax response. The protective activity of these Abs was established by passive transfer to anthrax-challenged mice. The ability of CpG ODN to accelerate and magnify the immune response to AVA suggests this strategy may contribute to the development of prophylactic and therapeutic vaccines against biothreat pathogens.
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Keywords: CpG oligonucleotide; Anthrax; Vaccine adjuvant

1. Introduction

Anthrax is caused by the gram-positive bacterium *Bacillus anthracis* [1,2]. Anthrax spores are highly resistant to environmental degradation and can remain infectious for decades. In the past, human exposure to anthrax resulted primarily from cutaneous contact with infected livestock [1,2]. Recently, spores formulated to readily infect the respiratory tract were intentionally released by bioterrorists in the US [3]. This caused immediate morbidity and mortality, and raised the specter of future exposure to these long-lived spores [3].

Vaccination is the least costly and most effective method of reducing susceptibility to anthrax infection and accelerating the development of protective immunity following pathogen exposure [1]. Neutralizing Abs directed against the bacteria's "protective antigen" (PA) reduce pathogenicity by preventing anthrax toxin from binding to host cells, inhibiting the germination of spores, and improving the phagocytosis/killing of spores by macrophages [4–7]. Unfortunately, the currently licensed human anthrax vaccine adsorbed (AVA) requires six vaccinations over 18 months followed by yearly boosters to induce and maintain protective IgG anti-PA titers [8,9]. In some vaccinees, this immunization regimen causes undesirable side effects [9].

Synthetic oligodeoxynucleotides (ODN) containing immunostimulatory "CpG motifs" can improve the immune response to co-administered antigens [10–12]. CpG ODN interact with Toll-like receptor 9 expressed by B cells and plasmacytoid dendritic cells [13–16], improving antigen presentation and triggering the production of chemokines and Th1 and pro-inflammatory cytokines (including IFNγ, IL-6, IL-12, IL-18 and TNFα) [13,14,17,18]. In mice, CpG ODN have been shown to boost the protective efficacy of vaccines against bacterial, viral and parasitic pathogens [19–23]. However, due to evolutionary divergence in CpG recognition between species, ODN that are highly active in rodents may be poorly immunostimulatory in primates [24–26]. Thus, pre-clinical studies to examine whether CpG ODN can accelerate and boost the immune response elictied by AVA must be conducted in a relevant primate model. This work provides evidence that co-administering GMP-grade CpG ODN with AVA to rhesus macaques increases the rapidity, titer, affinity, and protective efficacy of their resultant IgG anti-PA response.

Abbreviations: rPA, recombinant PA antigen; PA, protective antigen; AVA, anthrax vaccine adsorbed (licensed human anthrax vaccine); ODN, oligodeoxynucleotide

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2. Materials and methods

2.1. Reagents

The phosphorothioate ODN mixture containing equimolar amounts of ATCGACTCTCGAGCGTTCTC, TCGTTCG TTCTC and TCGAGCGTTCTC was synthesized at the Center for Biologics Core Facility. ODN 7909 was provided by Coley Pharmaceuticals. All ODN had less than <0.1 EU of endotoxin/mg of DNA as assessed by a Limulus amebocyte lysate assay (QCL-1000, BioWhittaker). AVA was obtained from BioPort Corporation (East Lansing, MI).

The attenuated non-encapsulated Sterne strain of anthrax was obtained from the culture collection of the United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD. Spores were prepared and stored as previously described [27].

2.2. Animals

All animal studies were ACUC approved and were conducted in AAALAC accredited facilities. Animals were monitored daily by veterinarians. Specific pathogen-free A/J mice were obtained from the Jackson Laboratories (Bar Harbor, ME) and housed in sterile micro-isolator cages in a barrier environment.

In the first experiment, rhesus macaques (3–5 kg, $N = 6$ per group) were immunized s.c. at 0 and 6 weeks with 0.5 ml of AVA plus 250 μg of an equimolar mixture of 3 CpG ODN (described below), and then "challenged" with 10^5 Sterne strain anthrax spores when serum anti-PA titers returned to baseline at week 26. Serum Ab levels were monitored 3 weeks post-immunization and 2 weeks post-boost per challenge. In the second experiment, macaques (3–5 kg, $N = 5$ per group) were immunized s.c. with 0.5 ml of AVA plus 500 ug of ODN 7909 or the same mixture of 3 CpG ODN mentioned above. Animals were bled at least weekly for 6 weeks. All treatments were administered and peripheral blood samples obtained from ketamine anesthetized animals (10 mg/kg, Ketaject, Phoenix Pharmaceuticals, St. Joseph, MD).

2.3. Anti-PA ELISA and avidity assays

Anti-PA titers were measured by coating 96-well Immulon 2 microtiter plates (Thermo LabSystems, Franklin MA) with 1 μg/ml of recombinant PA (rPA) (produced at USAMRIID as previously described [28]). Serum samples were serially diluted in PBS plus 5% non-fat dry milk and incubated on these plates for 1 h at RT. The plates were washed, and for avidity assays overlaid for 15 min with 200 μl of 6 M urea. Bound Ab was detected using peroxidase-conjugated goat anti-human IgG (Kirkegaard & Perry, Gaithersburg, MD) followed by ABTS substrate (Kirkegaard & Perry). Absorbance values were measured at 405 nm, and Ab titers calculated from the linear portion of the titration curve containing at least three contiguous dilutions ($R^2 > 0.9800$). Ab titers represent the reciprocal of the dilution that resulted in an absorbance value of 0.400, which was approximately twice background levels. For avidity comparisons, titers were determined by comparison to a standard curve generated using high-titered anti-PA serum. All samples were analyzed in triplicate.

2.4. Serum transfer study

Serum from all monkeys in each treatment group was pooled. One hundred microliters of pooled serum was injected i.p. into 6-week-old male A/J mice ($N = 20$ per group). The following day, mice were challenged i.p. with 500 μl of PBS containing 30 LD_{50} Sterne strain anthrax spores. Mice were monitored daily for 2 weeks, and time to death was recorded.

2.5. Toxin neutralization assay

RAW264.7 cells were plated overnight in 96-well microtiter plates at a concentration of 30,000 cells per well. Serum from vaccinated monkeys was serially diluted in complete medium (starting at 1:20) and incubated with 100 ng/ml rPA plus 100 ng/ml lethal factor (Research Diagnostics Inc., Flanders NJ) for 1 h at 37 °C. This mixture was added to the cells for 4 h at 37 °C. Ten microliters of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide, 5 mg/ml in PBS, Sigma, St. Louis MO) was added for 2 h, followed by lysis buffer (25 g SDS in 50% DMF, pH 4.7) to dissolve the formazan crystals. Absorbance values were read at 570 with a reference filter of 690.

2.6. Statistical analysis

Differences in serum anti-PA Ab titers were evaluated by multiple regression ANOVA. Differences in survival were evaluated using Chi-square analysis of Kaplan–Meier curves.

3. Results

3.1. CpG ODN increase the speed and titer of the IgG anti-PA Ab response induced by AVA immunization

Initial studies examined the effect of co-administering a mixture of CpG ODN [29] with AVA on the resultant immune response. The production of serum IgG anti-PA Abs was monitored, since such Abs are necessary and sufficient to protect against anthrax infection [1]. As seen in Fig. 1, adding CpG ODN to AVA increased the primary and secondary IgG response by two- to three-fold when compared to AVA alone ($P < 0.02$). At week 26, when Ab titers had

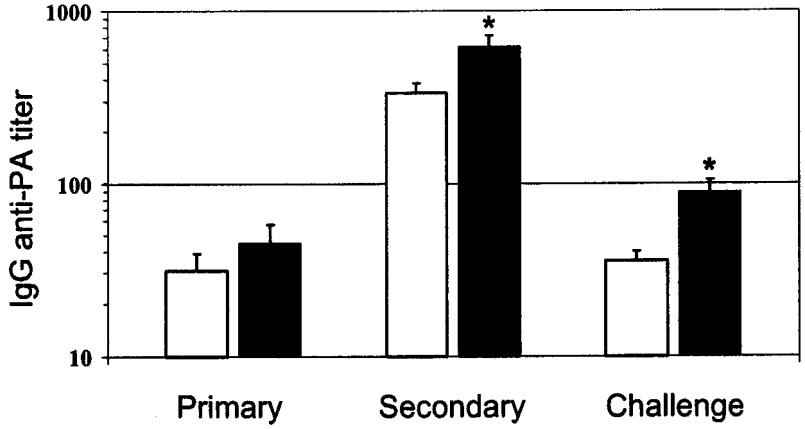

Fig. 1. Rhesus macaques (6 per group) were immunized s.c. with 0.5 ml of AVA alone (□) or combined with 250 ug of a mixture of the 3 CpG ODN described in Section 2 (■). Animals were boosted with the same material at week 6, and challenged with 10^5 Sterne strain anthrax spores at week 26. IgG anti-PA titers were measured in the serum of each animal 3 weeks post-immunization and 2 weeks post-boost per challenge. Results represent the mean ± S.E.M. of these titers. (*) Significantly higher IgG anti-PA titer at that time point, compared to animals immunized with AVA alone, $P < 0.05$.

fallen to near baseline levels, the animals were challenged with the attenuated Sterne strain of anthrax. The resultant IgG anti-PA response was significantly higher in macaques primed and boosted with AVA plus CpG ODN ($P < 0.01$).

The quality of a vaccine-adjuvant combination is reflected by both the avidity and titer of the Abs induced. To assess the avidity of the IgG anti-PA Abs, their ability to remain bound to PA in the presence of 6 M urea was evaluated [30,31]. As expected, affinity-matured Abs induced by secondary immunization were significantly more avid than those elicited by primary immunization (Fig. 2, $P < 0.01$). The avidity of the secondary anti-PA response of macaques immunized with AVA + CpG ODN was significantly higher than that of animals immunized with AVA alone ($P < 0.01$).

Based on this evidence that CpG ODN could improve the immunogenicity of AVA, a second experiment was conducted utilizing GMP-grade CpG ODN optimized for human use. Clinical grade CpG ODN 7909 (currently undergoing clinical trials for cancer therapy), the original CpG ODN mixture, or AVA alone, was administered to rhesus macaques. As seen in Fig. 3, the CpG ODN mixture again induced a stronger immune response than AVA alone. However, ODN 7909 triggered an even higher IgG anti-PA response. Starting by 11 days post-vaccination, ODN 7909 plus AVA was significantly more immunogenic than AVA alone ($P < 0.05$), generating a >3-fold higher IgG anti-PA response over the first month ($P < 0.01$).

3.2. Protective efficacy of the IgG anti-PA response

The critical measure of an antigen-adjuvant combination is its ability to induce protective immunity. Due to restrictions on the use of macaques in lethal anthrax challenge experiments, several alternative approaches were used to examine whether protective immunity was elicited by vaccination with AVA plus CpG ODN. In the first experiment,

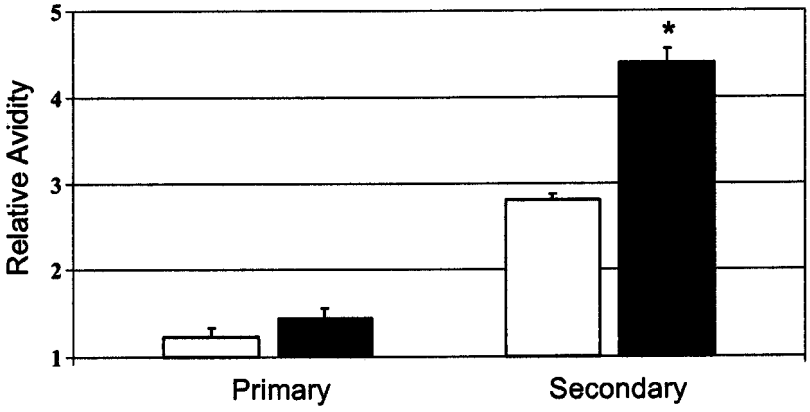

Fig. 2. The relative avidity ± S.E.M. of the IgG anti-PA Abs in the serum of animals immunized and boosted as described in Fig. 1 was determined by elution with 6 M urea. (*) Significantly greater avidity compared to animals immunized with AVA alone, $P < 0.03$.

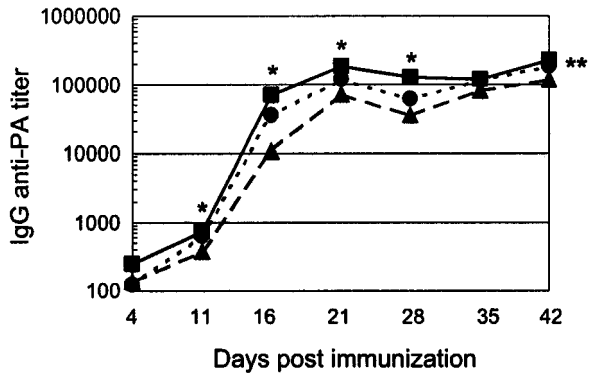

Fig. 3. Rhesus macaques (5 per group) were immunized s.c. with 0.5 ml of AVA alone (▲) or combined with 500 ug of the mixture of CpG ODN used in Fig. 1 (●) or ODN 7909 (■). IgG anti-PA titers were measured by ELISA in the serum of each animal at multiple time points post-immunization. Results represent the mean ± S.E.M. of these titers. (*) Significantly higher IgG anti-PA titer at that time point, compared to animals immunized with AVA alone, $P < 0.05$. (**) Significantly higher cumulative IgG anti-PA titer compared to animals immunized with AVA alone, $P < 0.01$.

immunized macaques were "challenged" with the attenuated (non-lethal) Sterne strain of *B. anthracis*. Challenged animals immunized with AVA plus CpG ODN mounted a 3-fold stronger immune response than animals immunized with AVA alone (Fig. 1, $P < 0.03$).

Second, the ability of serum Abs from immunized macaques to passively protect mice from anthrax challenge was explored. A/J mice are susceptible to the attenuated Sterne strain of anthrax, but are protected by neutralizing anti-PA Abs. Serum from pre-immune or immunized macaques was passively transferred to recipient mice (20 per group). As seen in Fig. 4, animals that received pre-immune serum or serum from AVA immunized macaques rapidly succumbed to challenge by 30 LD_{50} Sterne strain *B. Anthracis* spores. In contrast, serum from macaques immunized with AVA plus CpG ODN protected nearly half of recipient mice from lethal challenge ($P < 0.03$).

Table 1
Passive transfer of protection to naive recipient mice

Treatment	Donor	Recipient	Protection (%)
Serum neutralizing titer			
Pre-immunization	0 ± 0	10 ± 5	10
AVA alone	25 ± 5	43 ± 33	10
AVA + CpG ODN	434 ± 109*	2510 ± 1150*	45*

Rhesus macaques were immunized s.c. with 0.5 ml of AVA alone or combined with 500 μg of ODN 7909. Pre-immune serum, or serum 11 days post-vaccination, was pooled and injected i.p. into A/J mice (0.1 ml per recipient). The following day, mice were bled and then challenged with 30 LD_{50} of Sterne strain anthrax. The serum neutralizing titer of both donors (5 per group) and recipients ($N = 20$), and percent of recipients surviving challenge, are shown. Please note that mouse serum gave a higher background than macaque serum in this neutralizing assay.

* Significantly higher than AVA group, $P < 0.05$.

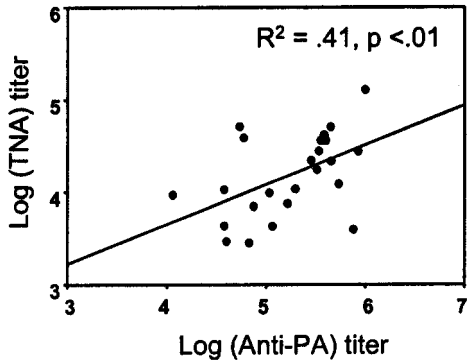

Fig. 5. IgG anti-PA titers and toxin neutralizing titers were monitored in sera from macaques immunized with AVA alone or AVA plus CpG ODN. Each data point represents the titer of a single serum measured by each assay.

These differences in susceptibility correlated with the titer of toxin neutralizing Abs present in donor serum transferred to recipient mice. Macaques immunized with AVA + ODN 7909 had on average a 17-fold higher toxin neutralizing titer than those immunized with AVA alone (Table 1, $P < 0.03$).

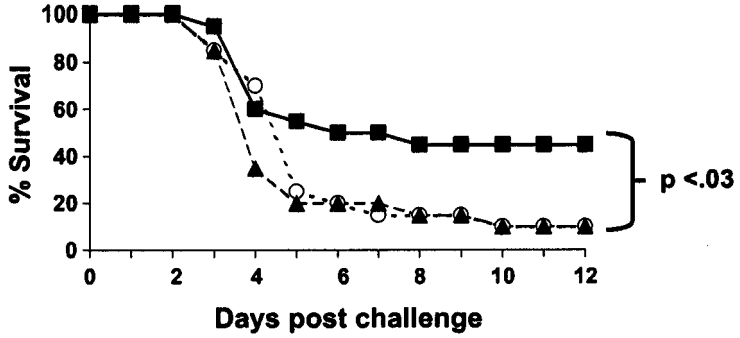

Fig. 4. Pre-immune serum (○), or serum from rhesus macaques vaccinated 11 days earlier with AVA alone (▲) or AVA + CpG ODN 7909 (■) was pooled and injected i.p. into A/J mice (0.1 ml per recipient). The following day, mice were challenged with 30 LD_{50} of Sterne strain anthrax. Results of two independent experiments involving at combined total of 20 recipients per treatment are shown.

A similar difference in neutralizing Ab levels was detected in the serum of recipient mice immediately prior to challenge (Table 1, $P < 0.05$). As expected, serum IgG anti-PA titers correlated closely with toxin neutralizing activity (Fig. 5, $R^2 = 0.41$, $P < 0.01$).

4. Discussion

CpG motifs present in bacterial DNA and synthetic oligonucleotides directly stimulate B cells and plasmacytoid dendritic cells that express Toll-like receptor 9 [15,16]. This initiates an immunostimulatory cascade that results in the functional maturation of professional antigen presenting cells and the production of Th1 and pro-inflammatory cytokines and chemokines [13,14,32,33].

Studies in mice demonstrated that CpG ODN could serve as immune adjuvants, significantly improving the immune response elicited by protein antigens and vaccines [21,22,34,35]. CpG ODN boosted the immune response elicited by vaccines against influenza, measles, hepatitis B surface Ag and tetanus toxoid by one to three orders of magnitude, while increasing the production of Th1 cytokines and the activity of antigen-specific CTL [34–39]. Due to evolutionary divergence in TLR 9 expression between species, CpG motifs optimized for activity in humans are less effective in mice [24–26]. Thus, pre-clinical studies of CpG ODN planned for human use are best performed in non-human primates [12,24–26,28]. Studies of orangutans, aotus monkeys, and rhesus macaques showed that CpG ODN boosted the immune response elicited by the hepatitis B vaccine and heat-killed leishmania vaccine by several fold [10–12].

The current work evaluated whether clinical grade CpG ODN (developed for use in cancer immunotherapy) could improve the immune response elicited by AVA (the licensed human anthrax vaccine). Results indicate that macaques immunized with AVA plus CpG ODN mounted a stronger immune response when challenged with Sterne strain anthrax spores (Fig. 1). The combination of CpG ODN plus AVA triggered a faster, higher avidity, and higher-titered immune response than vaccine alone, resulting in a significant improvement in protective immunity against anthrax (Fig. 4).

The induction of IgG anti-PA Abs is the most relevant measure of vaccine immunogenicity, since these Abs confer protection against infection [1]. Consistent with previous studies, serum levels of IgG anti-PA Abs correlated closely with toxin neutralizing activity, a surrogate marker for protective efficacy [1]. While antigen-specific T cell and cytokine responses may also be affected by CpG ODN [14,40], reagents to monitor anthrax-specific cellular responses in macaques are not available. Yet preliminary studies in mice showed that adding CpG ODN to AVA stimulated a predominantly Th1-biased immune response characterized by increased levels of IgG2a anti-PA antibodies (data not shown).

Current results demonstrate that the avidity of the secondary IgG anti-PA immune response was significantly improved by inclusion of CpG ODN (Fig. 2). This effect is consistent with the documented ability of CpG ODN to promote the functional maturation of professional APC [41]. In this context, ongoing studies suggest that other methods of targeting AVA to professional APC also results in higher-titered, more avid immune responses.

As with all novel therapies, the possibility of adverse side effects was considered. In previous studies, CpG ODN were safely administered to rodents and primates without adverse consequences [12,42]. In the current work, no serious local or systemic adverse reactions were observed in any of the macaques treated with CpG ODN plus AVA.

Vaccines targeting biothreat pathogens are typically designed for prophylactic (pre-exposure) use. However, vaccines capable of accelerating the development of protective immunity can be of therapeutic benefit to individuals exposed to biothreat pathogens (e.g., workers in anthrax-contaminated buildings) and/or for "ring vaccination" of individuals at sites of known infection. For these latter purposes, the capacity of CpG ODN to accelerate as well as increase the titer of anti-anthrax Abs is of particular interest. Our results demonstrate that co-administering CpG ODN with AVA generates high levels of toxin neutralizing antibodies very rapidly (exceeding AVA alone by 17-fold at 11 days post-immunization). Passive transfer of these serum antibodies protected nearly half of naive mice from challenge with 30 LD_{50} of anthrax spores (Fig. 4, $P < 0.03$ versus AVA alone). These findings support the further development of CpG ODN as an adjuvant for vaccines targeting biothreat pathogens.

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References

[1] Friedlander AM, Brachman PS. Anthrax. In: Plotkin SA, Mortimer EA, editors. Vaccines. Philadelphia (PA): WB Saunders; 1998. p. 729–39.

[2] Quinn CP, Turnbull PC. Anthrax. In: Ballow M, Sussman M, editors. Topley and Wilson's microbiology and microbial infections, 98 A.D. London: Collier. p. 799–818.

[3] Lane HC, Montagne JL, Fauci AS. Bioterrorism: a clear and present danger. Nat Med 2001;7(12):1271–3.

[4] Ivins BE, Welkos SL, Liffle SF, Crumrine MH, Nelson GO. Immunization against anthrax with *Bacilus anthracis* protective antigen combined with adjuvants. Infect Immun 1992;60:662–8.

[5] Welkos SL, Friedlander AM. Comparative safety and efficacy against *Bacillus anthracis* of protective antigen and live vaccines in mice. Microb Pathog 1988;5(2):127–39.
[6] Little SF, Ivins BE. Molecular pathogenesis of *Bacillus anthracis* infection. Microb Infect 1999;1(2):131–9.
[7] Welkos S, Little S, Friedlander A, Fritz D, Fellows P. The role of antibodies to *Bacillus anthracis* and anthrax toxin components in inhibiting the early stages of infection by anthrax spores. Microbiology 2001;147(Pt 6):1677–85.
[8] Pittman PR, Kim-Ahn G, Pifat DY, Coonan K, Gibbs P, Little S, et al. Anthrax vaccine: immunogenicity and safety of a dose-reduction, route-change comparison study in humans. Vaccine 2002;20(9–10):1412–20.
[9] Pittman PR, Gibbs PH, Cannon TL, Friedlander AM. Anthrax vaccine: short-term safety experience in humans. Vaccine 2001;20(5–6):972–8.
[10] Davis HL, Suparto II, Weeratna RR, Jumintarto DD, Chamzah SS, Ma'ruf AA, et al. CpG DNA overcomes hyporesponsiveness to hepatitis B vaccine in orangutans. Vaccine 2000;19(4/5):413–22.
[11] Jones TR, Obaldia N, Gramzinski RA, Charoenvit Y, Kolodny N, Kitov S, et al. Synthetic oligodeoxynucleotides containing CpG motifs enhance immunogenic vaccine in Aotus monkeys. Vaccine 1999;17(23–24):3065–71.
[12] Verthelyi D, Kenney RT, Seder RA, Gam AA, Friedag B, Klinman DM. CpG oligodeoxynucleotides as vaccine adjuvants in primates. J Immunol 2002;168(4):1659–63.
[13] Krieg AM, Yi A, Matson S, Waldschmidt TJ, Bishop GA, Teasdale R, et al. CpG motifs in bacterial DNA trigger direct B-cell activation. Nature 1995;374:546–8.
[14] Klinman DM, Yi A, Beaucage SL, Conover J, Krieg AM. CpG motifs expressed by bacterial DNA rapidly induce lymphocytes to secrete IL-6, IL-12 and IFNγ. Proc Natl Acad Sci USA 1996;93:2879–83.
[15] Takeshita F, Leifer CA, Gursel I, Ishii K, Takeshita S, Gursel M, et al. Cutting Edge: role of toll-like receptor 9 in CpG DNA-induced activation of human cells. J Immunol 2001;167(7):3555–8.
[16] Hemmi H, Takeuchi O, Kawai T, Sato S, Sanjo H, Matsumoto M, et al. A Toll-like receptor recognizes bacterial DNA. Nature 2000;408:740–5.
[17] Ballas ZD, Rasmussen WL, Krieg AM. Induction of NK activity in murine and human cells by CpG motifs in oligodeoxynucleotides and bacterial DNA. J Immunol 1996;157:1840–7.
[18] Halpern MD, Kurlander RJ, Pisetsky DS. Bacterial DNA induces murine interferon-gamma production by stimulation of IL-12 and tumor necrosis factor-alpha. Cell Immunol 1996;167:72–8.
[19] Klinman DM. Therapeutic applications of CpG-containing oligodeoxynucleotides. Antisense Nucleic Acid Drug Dev 1998;8:181–4.
[20] Lipford GB, Bauer M, Blank C, Reiter R, Wagner H, Heeg K. CpG-containing synthetic oligonucleotides promote B and cytotoxic T cell responses to protein antigen: a new class of vaccine adjuvants. Eur J Immunol 1997;27:2340–4.
[21] Davis HL. Use of CpG DNA for enhancing specific immune responses. Curr Top Microbiol Immunol 2000;247:171–84.
[22] Moldoveanu Z, Homan LL, Huang WQ, Krieg AM. CpG DNA, a novel immune enhancer for systemic and mucosal immunization with influenza virus. Vaccine 1999;16:1216–24.
[23] Krieg AM, Davis HL. Enhancing vaccines with immune stimulatory CpG DNA. Curr Opin Mol Ther 2001;3(1):15–24.
[24] Bauer M, Heeg K, Wagner H, Lipford GB. DNA activates human immune cells through a CpG sequence-dependent manner. Immunology 1999;97(4):699–705.
[25] Hartmann G, Krieg AM. Mechanism and function of a newly identified CpG DNA motif in human primary B cells. J Immunol 2000;164(1):944–52.
[26] Verthelyi D, Ishii KJ, Gursel M, Takeshita F, Klinman DM. Human peripheral blood cells differentially recognize and respond to two distinct CpG motifs. J Immunol 2001;166:2372–7.
[27] Ivins BE, Welkos SL, Knudson GB, Little sF. Immunization against anthrax with aromatic compound-dependent (Aro-) mutants of *Bacillus anthracis* and with recombinant strains of *Bacillus subtilis* that produce anthrax protective antigen. Infect Immun 1990;58(2):303–8.
[28] Ivins BE, Pitt ML, Fellows PF, Farchaus JW, Benner GE, Waag DM, et al. Comparative efficacy of experimental anthrax vaccine candidates against inhalation anthrax in rhesus macaques. Vaccine 1998;16(11-12):1141–8.
[29] Leifer CA, Verthelyi D, Klinman DM. Heterogeneity in the human response to immunostimulatory CpG oligodeoxynucleotides. J Immunother 2003;26(4):313–9.
[30] Eggers M, Bader U, Enders G. Combination of microneutralization and avidity assays: improved diagnosis of recent primary human cytomegalovirus infection in single serum sample of second trimester pregnancy. J Med Virol 2000;60(3):324–30.
[31] Cozon GJ, Ferrandiz J, Nebhi H, Wallon M, Peyron F. Estimation of the avidity of immunoglobulin G for routine diagnosis of chronic *Toxoplasma gondii* infection in pregnant women. Eur J Clin Microbiol Infect Dis 1998;17(1):32–6.
[32] Sparwasser T, Koch E, Vabulas RM, Heeg K, Lipford GB, Ellwart JW, et al. Bacterial DNA and immunostimulatory CpG oligonucleotides trigger maturation and activation of murine dendritic cells. Eur J Immunol 1998;28(6):2045–54.
[33] Roman M, Martin-Orozco E, Goodman JS, Nguyen M, Sato Y, Ronaghy A, et al. Immunostimulatory DNA sequences function as T helper-1 promoting adjuvants. Nat Med 1997;3:849–54.
[34] Brazolot Millan CL, Weeratna R, Krieg AM, Siegrist CA, Davis HL. CpG DNA can induce strong Th1 humoral and cell-mediated immune responses against hepatitis B surface antigen in young mice. Proc Natl Acad Sci USA 1998;95:15553–8.
[35] McCluskie MJ, Davis HL. CpG DNA is a potent enhancer of systemic and mucosal immune responses against hepatitis B surface antigen with intranasal administration to mice. J Immunol 1998;161:4463–6.
[36] Moldoveanu Z, Love-Homan L, Huang WQ, Krieg AM. CpG DNA, a novel immune enhancer for systemic and mucosal immunization with influenza virus. Vaccine 1998;16:1216–24.
[37] Branda RF, Moore AL, Lafayette AR, Mathews L, Hong R, Zon G, et al. Amplification of antibody production by phosphorothioate oligodeoxynucleotides. J Lab Clin Med 1996;128:329–38.
[38] Kovarik J, Bozzotti P, Love-Homan L, Pihlgren M, Davis HL, Lambert P-H AM, et al. CpG oligonucleotides can cirmcuvent the TH2 polorization of neonatal responses to vaccines but fail to fully redirect TH2 responses established by neonatal priming. J Immunol 1999;162:1611–7.
[39] Davis HL, Weeranta R, Waldschmidt TJ, Tygrett L, Schorr J, Krieg AM. CpG DNA is a potent enhancer of specific immunity in mice immunized with recombinant hepatitis B surface antigen. J Immunol 1998;160:870–6.
[40] Sun S, Zhang X, Tough DF, Sprent J. Type I interferon-mediated stimulation of T cells by CpG DNA. J Exp Med 1998;188:2335–42.
[41] Gursel M, Verthelyi D, Gursel I, Ishii KJ, Klinman DM. Differential and competitive activation of human immune cells by distinct classes of CpG oligodeoxynucleotides. J Leukoc Biol 2002;71:813–20.
[42] Klinman DM, Conover J, Coban C. Repeated administration of synthetic oligodeoxynucleotides expressing CpG motifs provides long-term protection against bacterial infection. Infect Immun 1999;67:5658–63.

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Effect of aluminum hydroxide adjuvant and formaldehyde in the formulation of rPA anthrax vaccine☆

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Abstract

The serological response and efficacy of *Bacillus anthracis* recombinant protective antigen (rPA) vaccines formulated with aluminum hydroxide adjuvant, either with or without formaldehyde, were evaluated in rabbits. Rabbits that had been injected with a single dose of 25 μg of rPA adsorbed to 500 μg of aluminum in aluminum hydroxide gel (Alhydrogel) had a significantly higher quantitative anti-rPA IgG ELISA titers ($p < 0.0001$) and toxin neutralizing antibody (TNA) assay titers ($p < 0.0001$) than rabbits tested at the next lowest concentration of aluminum (158 μg). Rabbits injected with two doses of 50 μg of rPA formulated with 500 μg of aluminum also had significantly higher serological responses, as measured by a quantitative anti-rPA IgG ELISA ($p < 0.0001$) and TNA assay ($p < 0.0001$), than sera from rabbits injected with a rPA vaccine formulated without adjuvant. Short-term protection against an aerosol spore challenge (448 LD_{50}), however, was not significantly different between the two groups (12/12 and 11/12, respectively). Rabbits injected with a single dose of 50 μg of rPA formulated with 500 μg of aluminum and 0.2% formaldehyde had significantly higher ELISA ($p < 0.0001$) and TNA assay ($p < 0.0001$) titers than rabbits that had been injected with a rPA vaccine formulated with adjuvant but without formaldehyde. Short-term protection against a 125 LD_{50} parenteral spore challenge, however, was not significantly different between the two groups (14/24 and 9/24, respectively; $p = 0.2476$). Under the conditions tested in the rabbit animal model, significantly higher serological responses were observed in rabbits that had been injected with rPA formulated with aluminum hydroxide gel adjuvant and formaldehyde. However, differences in short-term efficacy were not observed.

Keywords: Bacillus anthracis PA vaccine formulation; Rabbit animal model; Aluminum hydroxide gel adjuvant; Formaldehyde

1. Introduction

Anthrax is an infection that may result after exposure to spores of *Bacillus anthracis* by the cutaneous, gastrointestinal, or aerosol routes and may be characterized by an extensive bacteremia and toxemia. A small number of cutaneous infections, which are usually self-limiting, as well as gastrointestinal and aerosol infections, are life threatening. The bacteremia is facilitated by the expression of a poly-D-glutamic acid capsule which interferes with phagocytosis of the vegetative bacterium. Toxemia is the result of two separate binary toxins, lethal toxin (LeTx) and edema toxin. A central component of both toxins is protective antigen (PA). After PA binds to a cellular receptor,

☆ Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, National Research Council, 1996. The facility where this research was conducted is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. Opinions, interpretations, conclusions, and recommendations are those of the author and are not necessarily endorsed by the U.S. Army. The research described herein was sponsored by the U.S. Army Medical Research and Materiel Command, Project 02-4-CC-008.

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it undergoes proteolysis by the cell-surface protease resulting in a receptor-bound fragment, PA63. The PA63 molecules form a heptameric pre-pore which complexes with the enzymatic components, either lethal factor (LF), forming LeTx, or edema factor (EF), to form edema toxin. The toxin complex is then endocytosed by the cell and within the endocytic compartment the pre-pore undergoes an acidic pH-dependent conformational rearrangement that allows translocation of LF and EF into the cytosol [1].

Anthrax vaccine adsorbed, Biothrax (AVA Biothrax; BioPort Corporation, Lansing, MI) is the current vaccine licensed for human use against exposure to *B. anthracis* spores in the U.S. The vaccine is prepared by adsorbing filtered culture supernatant fluids of the V770-NPR-1 strain of *B. anthracis* to an aluminum hydroxide gel. The major protective antigen in AVA BioThrax is PA [2–5], the central component of the *B. anthracis* exotoxins. Also present in the vaccine are LF and undefined bacterial proteins, which are present in the filtered culture supernatant and which are also adsorbed onto the adjuvant [6]. In addition to the adjuvant, AVA Biothrax is formulated to contain 25 μg/ml of benzethonium chloride as a preservative and 100 μg/ml of formaldehyde as a stabilizer. Several concerns have been raised in regards to the vaccine, including the lot-to-lot variation in the amounts of PA in the vaccine [6] and occasional reactogenicity after vaccination, which may be related to the presence of uncharacterized components and possibly formaldehyde [7–9] that have served as a stimulus to develop a more fully characterized vaccine. Several studies have demonstrated the efficacy of PA in vaccines to protect against anthrax intoxication or infection [10–13]. The importance of anti-PA serum also has been shown in the identification of in vitro correlates of immunity [14–17] and in passive antibody studies [18–20]. This report evaluates the role of the aluminum hydroxide gel adjuvant and the excepient formaldehyde in the formulation of rPA vaccines in the rabbit model using in vitro surrogate markers (the quantitative anti-rPA IgG ELISA and toxin neutralizing antibody (TNA) assay) and efficacy studies.

2. Materials and methods

2.1. Animals

An equal number of male and female New Zealand white (NZW) rabbits (3.0–3.5 kg) (Covance Research Products, Denver, Penn.) were used for each experiment. The animals received food and water *ad libitum*. Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, National Research Council, 1996. The facility where this research was conducted is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

2.2. Preparation of rPA vaccine, vaccination, and challenge

Recombinant PA (rPA), expressed in a *B. anthracis* background [21,22], was manufactured as a cGMP lot by the Biopharmaceutical Production Facility at NCI-FCRC (Frederick, MD) using a modification of a reported procedure [23]. The same lot of rPA was used throughout these experiments for vaccinations and serological analysis of antibody response. LF was prepared by chromatographic separation from V770-NP1-R culture supernatants as previously described [24].

The effect of aluminum hydroxide gel adjuvant on the serological response to rPA was evaluated by adsorbing 25 μg of rPA diluted in Dulbecco's phosphate buffered saline without Ca^{2+} or Mg^{2+} (PBS) to various concentrations of aluminum (500–15.8 μg per 0.5 ml dose in half-log dilutions) in Alhydrogel (2% Al_2O_3; HCl Biosector, Frederikssund, Denmark) for 20–24 h at 4 °C before use. NZW rabbits were inoculated intramuscularly (i.m.) with a single dose in 0.5 ml volumes and were bled weekly. The immunological status of the rabbits was measured by using a quantitative anti-rPA IgG ELISA and TNA assay [15].

The effect of adjuvant (Alhydrogel) on the serological response and protection was evaluated in NZW rabbits, which were inoculated i.m. at 0 and 4 weeks with 50 μg of rPA formulated with either 500 μg of aluminum per injection, final concentration, or without adjuvant in 0.5-ml volumes. Control rabbits were injected with PBS and Alhydrogel. Rabbits were bled every other week to determine serological titers to PA (quantitative anti-rPA IgG ELISA and TNA assay). At 10 weeks, rabbits were exposed to a small-particle aerosol in a modified Henderson exposure system contained within a Class III biological safety cabinet to the head only (nose and mouth) with a lethal dose of spores from the Ames isolate of *B. anthracis* [25]. Inhaled doses were calculated using the aerosol exposure concentration obtained from plate counts from the all-glass impinger which continuously sampled the test atmosphere during the 10 min exposure time and the respiratory minute volume for each animal measured by plethysmography. Spores were prepared as previously described [25]. Survival was noted for 21 days after challenge. The aerosol LD_{50} of Ames spores in NZW rabbits is 1.1×10^5 spores [25]. The average inhaled dose (average $LD_{50} \pm$ S.D.) of spores was 448 ± 214.6 LD_{50}.

The effect of formaldehyde on the immune response in NZW rabbits after injection of a rPA vaccine preparation was also evaluated. Vaccine preparations were formulated with 50 μg of rPA adsorbed to 500 μg of aluminum in Alhydrogel with or without 0.02% formaldehyde. Rabbits were inoculated i.m. with a single dose of vaccine. Control rabbits were injected with PBS and Alhydrogel. Rabbits were bled every other week after inoculation, and the sera were tested by a quantitative anti-rPA IgG ELISA and TNA assay. Rabbits were challenged subcutaneously (s.c.) at week 6 with 125

LD_{50} spores from the Ames isolate of *B. anthracis* (actual count was 1.9×10^5 spores). Spores were prepared as previously described [25]. Survival was noted for 21 days after challenge. The s.c. LD_{50} of Ames spores in NZW rabbits is 1.5×10^3 spores.

2.3. Serological analysis of antibodies

Blood was collected periodically for analysis of serum antibodies by a quantitative anti-rPA IgG ELISA and TNA assay [15]. ELISA titers were determined by interpolating the average absorbance value for triplicate wells of each sample with the absorbance values of a standard curve generated from seven dilutions of affinity purified rabbit anti-rPA IgG by linear regression analysis and reported as micrograms of anti-rPA IgG per ml (KC4 software, BioTek Instruments, Winooski, VT) [15]. Titers were presented as the geometric mean and ×/÷ standard error of the geometric mean (S.E.M.). For the TNA assay, the average absorbance value of triplicate wells for each test sample dilution, less the average absorbance value of triplicate wells incubated with LeTx, was divided by the average absorbance value of control wells that contained only medium, less the average absorbance value of triplicate wells incubated with LeTx, and the ratio multiplied by 100 to obtain the percent viability of the test wells compared to the control wells:

$$\%\text{Control} = \left(\frac{\text{sample avg} - \text{LeTx avg}}{\text{medium control avg} - \text{LeTx avg}} \right) \times 100.$$

The percent control values were plotted against each respective test dilution using a four-parameter logistic equation algorithm. TNA assay titers were expressed as the reciprocal of the dilution of antiserum at which neutralization of the cytotoxic activity of LeTx on J774A.1 cells was half-maximal (50%; ED_{50}) using XLfit software (IDBS Inc., Emeryville, CA). Titers were presented as the geometric mean and ×/÷ S.E.M.

2.4. Statistical analysis

Log_{10} transformations were applied to all ELISA and TNA assay ED_{50} titers except for TNA assay ED_{50} titers from results presented below in Section 3.1. After transformation, the dependent variable met assumptions of normality and homogeneity of variance. Mixed model analysis of variance (RM-ANOVA) or ANOVA were used to compare titers over time and between challenge groups. Fisher exact tests and Chi-square tests for proportions were used to compare survival rates, which are the ratio between survivors and the total number of test animals at the end of the study. Kaplan-Meier survival analysis, which is a plot of the percent survival as a function of time, was used to compare survival curves between groups. Analyses were conducted using SAS Version 8.2 (SAS Institute Inc., SAS OnlineDoc, Version 8, Cary, NC).

3. Results and discussion

3.1. Dose effect of Alhydrogel adjuvant on serological responses

During the development of an anthrax vaccine based upon rPA, numerous adjuvants were evaluated for their efficacy in guinea pigs [5,26] and non-human primates [12]. Aluminum-based adjuvants however, are the only class of adjuvants that have been approved for use in humans. AVA Biothrax is prepared from V770-NP1-R filtered culture supernatants adsorbed to 650 μg of aluminum hydroxide gel per 0.5 ml dose [6]. The British vaccine approved for human use consists of filtered culture supernatants of the Sterne strain of *B. anthracis* precipitated with aluminum phosphate gel (alum) [27]. Although the maximal amount of aluminum that is allowed for the U.S. vaccine is 850 μg per dose, the recommended maximum concentration for anthrax vaccines based upon rPA is 500 μg per dose. Our first experiment examined the serological response of NZW rabbits injected with a rPA vaccine formulated with various concentrations of aluminum present in aluminum hydroxide gel (Alhydrogel). Animals that were injected with a single dose of 25 μg of rPA adsorbed to 500 μg of aluminum per 0.5 ml dose had a geometric mean peak ELISA titer at week 2 of 31.0 μg of anti-rPA IgG per ml (Table 1). At a half-log lower dose of adjuvant (158 μg of aluminum), the geometric mean week 2 anti-rPA ELISA titer dropped significantly ($p < 0.0001$) by eight-fold to 4.0 μg of anti-rPA IgG per ml. Each half-log lower concentration of aluminum adjuvant in the rPA vaccine preparations resulted in two-fold decreases in week 2 anti-rPA ELISA titers (Table 1). The geometric mean TNA assay ED_{50} titer at week 2 for rabbits that had been injected with a single dose of 25 μg of rPA adsorbed to 500 μg of aluminum was 360 (Table 1). The TNA assay ED_{50} titer dropped significantly ($p < 0.0001$) by five-fold to 69.4 for rabbits injected with 158 μg of aluminum per dose (Table 1). At lower concentrations of adjuvant, a decrease in TNA assay ED_{50} titers were also observed (Table 1). It would appear that for a maximal serological response for rabbits, the maximum recommended concentration of 500 μg of aluminum per dose was the most effective among the doses tested.

3.2. Effect of Alhydrogel on serological response and protection

The effect of the adjuvant on the serological response and protective efficacy was examined in NZW rabbits that were injected with rPA vaccines formulated either with or without Alhydrogel. Rabbits were inoculated with two doses of 50 μg of rPA at 0 and 4 weeks. For one group, rPA was adsorbed to aluminum hydroxide adjuvant at 500 μg of aluminum per dose while the second group did not receive adjuvant. Control rabbits were injected with PBS and Alhydrogel. Titers peaked 2 weeks after the second dose of vaccine on week 6 for both groups (Tables 2a and 2b). The anti-rPA IgG

Table 1
Quantitative anti-rPA IgG ELISA and TNA assay ED_{50} titers of rabbits after a single inoculation with 25 μg of rPA adsorbed to various concentrations of aluminum

Aluminum concentration (μg)	Quantitative anti-rPA IgG ELISA titer[a]				TNA assay ED_{50} titer[b]
	Week 1	Week 2	Week 3	Week 4	Week 2
500	2.5 (1.409)	31.0 (1.209)	18.2 (1.158)	14.2 (1.195)	360 (1.133)
158	0.56 (1.344)	4.0 (1.372)	3.5 (1.302)	3.2 (1.294)	69.4 (1.649)
50	0.49 (1.075)	2.3 (1.251)	2.1 (1.321)	2.1 (1.217)	17.0 (1.782)
15.8	0.51 (1.272)	1.1 (1.262)	0.87 (1.233)	1.1 (1.251)	7.0 (1.641)
0	0.15 (1.213)	BLQ[c]	0.10 (1.254)	0.09 (1.085)	1.0 (1.00)

[a] Titer expressed as μg of anti-rPA IgG per ml and standard error of the mean (S.E.M.) in parenthesis.
[b] Titer expressed as the reciprocal of the dilution of antiserum at which neutralization of the cytotoxic activity of LeTx on J774A.1 cells was half-maximal (50%; ED_{50}) and S.E.M. in parenthesis. If the TNA assay ED_{50} titer could not be extrapolated from the 4-parameter logistic regression curve, the value was arbitrarily assigned a value of '1.0'. The starting dilution for the TNA assay was 1:50.
[c] BLQ, below the limit of quantitation which was 0.072 μg/ml of IgG, the concentration of the lowest standard (1.44 ng/ml of IgG) multiplied by the lowest starting concentration of the sample (1:50) of the ELISA.

Table 2a
Geometric mean quantitative anti-rPA IgG ELISA titers and survival ratio of rabbits inoculated with 50 μg of rPA formulated with or without aluminum hydroxide gel adjuvant[a]

Vaccination group	Anti-rPA IgG ELISA titer[b]						Survival ratio[a]
	Week 2	Week 4	Week 6	Week 8	Week 10	Post-challenge	
With adjuvant	14.1 (1.340)	8.1 (1.330)	342 (1.154)	207 (1.164)	158 (1.134)	322 (1.130)	12/12
Without adjuvant	1.5 (1.311)	1.2 (1.220)	72.0 (1.259)	50.1 (1.248)	31.8 (1.184)	511 (1.176)	11/12
Control	0.44 (1.392)	0.16 (1.616)	0.18 (1.260)	0.09 (1.138)	0.14 (1.741)	na[c]	0/4

[a] NZW rabbits were inoculated i.m. at 0 and 4 weeks with rPA vaccine formulated either with or without 500 μg of aluminum adjuvant (Alhydogel). Rabbits were challenged on week 10 by the aerosol route with spores from the Ames isolate of *B. anthracis*.
[b] Titer expressed as μg of anti-rPA IgG per ml and S.E.M. in parenthesis.
[c] na, sample not available.

ELISA antibody responses (Table 2a) and TNA assay ED_{50} titers (Table 2b) were significantly higher in rabbits that were injected with rPA adsorbed to aluminum hydroxide adjuvant than in rabbits that were injected with rPA without aluminum hydroxide gel adjuvant. There was a significant difference in ELISA titers between the two groups ($F(1,108) = 161.75$, $p < 0.0001$) over time ($F(4,108) = 143.45$, $p < 0.0001$) and at each week tested (p values $p < 0.0001$ for each week). There also was a significant difference in TNA assay ED_{50} titers between the two groups ($F(4,110) = 145.43$, $p < 0.0001$) over time ($F(4,110) = 118.60$, $p < 0.0001$) and at each week tested (p values $p < 0.004$ for each week). Protection against an aerosol exposure to *B. anthracis* spores was measured 6 weeks after the booster inoculation (week 10). Rabbits were challenged by aerosols consisting of spores of the Ames isolate of *B. anthracis*. The average inhaled dose (average $LD_{50} \pm$ S.D.) of spores that was measured for the rabbits was 448 ± 214.6 LD_{50}. Rabbits that were inoculated with two doses of rPA adsorbed to Alhydrogel were fully protected against the aerosol challenge (100%; 12/12), whereas 92% of rabbits (11/12) inoculated with rPA without adjuvant were protected (Table 2a). None of the control rabbits survived the challenge (0%; 0/4). The serological responses of the rabbits from each group were also compared 21 days after challenge. The post-challenge quantitative anti-rPA IgG ELISA titers and TNA assay ED_{50} titers from the vaccination group receiving rPA adsorbed to Alhydrogel (322 μg anti-rPA IgG per ml and 6151, respectively) were similar to quantitative anti-rPA IgG ELISA titers and TNA assay ED_{50} titers measured 2 weeks after the booster injection (342 μg anti-rPA IgG per ml and 4367, respectively). The post-challenge quantitative anti-rPA IgG ELISA titers and TNA

Table 2b
Geometric mean TNA assay ED_{50} titers of rabbits inoculated with 50 μg of rPA formulated with or without aluminum hydroxide gel adjuvant[a]

Vaccination group	TNA assay ED_{50} titer[b]					
	Week 2	Week 4	Week 6	Week 8	Week 10	Post-challenge
With adjuvant	203 (1.360)	74.5 (1.435)	4367 (1.125)	2029 (1.100)	1065 (1.100)	6151 (1.146)
Without adjuvant	4.5 (1.766)	2.3 (1.489)	1090 (1.218)	542 (1.226)	312 (1.235)	10422 (1.183)
Control	1.3 (1.172)	1.0 (1.00)	2.0 (1.478)	4.3 (1.626)	1.3 (1.147)	na[c]

[a] NZW rabbits were inoculated i.m. at 0 and 4 weeks with rPA vaccine formulated either with or without 500 μg of aluminum adjuvant (Alhydogel).
[b] Titer expressed as the reciprocal of the dilution of antiserum at which neutralization of the cytotoxic activity of LeTx on J774A.1 cells was half-maximal (50%; ED_{50}) and S.E.M. in parenthesis. If the TNA assay ED_{50} titer could not be extrapolated from the 4-parameter logistic regression curve, the value was arbitrarily assigned a value of '1.0'. The starting dilution for the TNA assay was 1:50.
[c] na, sample not available.

assay ED_{50} titers however, from the rPA vaccination group formulated without Alhydrogel (511 μg anti-rPA IgG per ml and 10,422, respectively) were much higher than the quantitative anti-rPA IgG ELISA titers and TNA assay ED_{50} titers measured 2 weeks after the booster injection (72 μg anti-rPA IgG per ml and 1090, respectively). A significant increase in ELISA or TNA assay titers suggests an absence of sterile immunity. Rabbits inoculated with rPA adsorbed to Alhydrogel had post-challenge ELISA titers and TNA assay ED_{50} titers that were two-fold higher ($p = 0.0014$) and six-fold higher ($p < 0.0001$), respectively, than those measured at week 10. Significantly higher titers were measured also in rabbits that had been inoculated with rPA without Alhydrogel, which had a seven-fold increase in week 10 ELISA titers ($p < 0.0001$) and a 33-fold increase in week 10 TNA assay ED_{50} titers ($p < 0.0001$) post-challenge. The difference in post-challenge ELISA titers and TNA assay ED_{50} titers between the two groups was significantly different ($F(1,21) = 5.28$, $p = 0.0319$ and ($F(1,21) = 6.05$, $p = 0.0227$, respectively). In addition to resulting in higher quantitative anti-rPA IgG ELISA titer and TNA assay ED_{50} titers, the formulation of rPA with aluminum adjuvant in the vaccine resulted in a lower increase in the post-challenge serological responses than what was observed in rabbits that had been injected with the rPA vaccine formulated without aluminum hydroxide gel adjuvant (Tables 2a and 2b).

3.3. Effect of formaldehyde on serological response and protection

AVA Biothrax is formulated to contain 0.01% formaldehyde as a stabilizer and 0.0025% benzethonium chloride as a preservative [6]. Studies that had been conducted in evaluating rPA vaccine preparations, including serological correlates of immunity in rabbits [15], potency assay [28], and duration of immunity in rabbits [29], were not formulated with components other than aluminum hydroxide gel adjuvant. The serological response and efficacy of vaccines containing 50 μg of rPA adsorbed to Alhydrogel (500 μg of aluminum) and formulated either with 0.02% formaldehyde or without formaldehyde were compared in rabbits inoculated i.m. with a single injection of vaccine (Table 3). Geometric mean anti-rPA IgG ELISA titers between the two groups were significantly different at week 2, ($p = 0.0001$) and week 4 ($p = 0.0002$) but not at week 6 ($p = 0.0652$). Similarly, geometric mean TNA assay ED_{50} titers between the two groups were significantly different at week 2 ($p < 0.0001$) and week 4 ($p = 0.0003$) but not at week 6 ($p = 0.1119$). Rabbits were challenged s.c. at 6 weeks with spores from the Ames isolate of *B. anthracis*. The rPA vaccine formulated with 500 μg of aluminum and 0.02% formaldehyde protected 58% of rabbits (14/24) against the parenteral challenge, while 37.5% of rabbits (9/24) inoculated with rPA formulated with 500 μg of aluminum but without formaldehyde were protected. None of the control rabbits survived the challenge (0%; 0/4). The s.c. route of challenge was evaluated because it provided greater control of the number of spores that were in the challenge. Neither the difference in survival rates ($p = 0.1486$), survival curves ($\chi^2(1) = 1.62$, $p = 0.2037$), nor mean time-to-death (4.2 days for rPA with formaldehyde and 4.5 days for rPA without formaldehyde; $p = 0.7959$) between the two groups was significant. Our data do not suggest that the inclusion of formaldehyde is a necessary additive to the rPA vaccine. From these results, it appears that formaldehyde's action, in addition to acting as a stabilizer, may also be one of an adjuvant. Studies from a booster injection were not investigated.

Aluminum compounds, aluminum hydroxide ($Al(OH)_3$), aluminum phosphate ($AlPO_4$), and alum ($KAl(SO_4)_2$), are the only adjuvants currently approved for use in human vaccines and are used in the formulation of many veterinary vaccines. The currently licensed U.S. anthrax vaccine, AVA Biothrax, is prepared by adsorbing filtered culture supernatants of the *B. anthracis* V770-NP1-R strain to aluminum hydroxide gel [6]. The current British anthrax vaccine, AVP, is prepared by precipitating filtered culture supernatants of the *B. anthracis* Sterne strain with aluminum phosphate (alum) [27]. AVP contains more LF and EF than AVA Biothrax as measured by antibody response to these components [30]. Studies have shown that AVA Biothrax provides

Table 3
Survival ratio, quantitative anti-rPA IgG ELISA titer, and TNA assay ED_{50} titer of rabbits injected with 50 μg of rPA formulated with or without formaldehyde[a]

Vaccination Group	Survival ratio[b]	Anti-rPA IgG titer[c]			TNA assay titer[d]		
		Week 2	Week 4	Week 6	Week 2	Week 4	Week 6
With formaldehyde	14/24	53.9 (1.188)	36.3 (1.180)	12.7 (1.189)	611 (1.150)	289 (1.148)	157 (1.171)
Without formaldehyde	9/24	18.6 (1.235)	12.9 (1.217)	7.7 (1.239)	145 (1.404	86.6 (1.367)	93.4 (1.210)
Control	0/4	BLQ[e]	BLQ	BLQ	1.3 (1.316)	2.3 (2.300)	1.0 (1.000)

[a] Rabbits were inoculated with a single dose of 50 μg of rPA vaccine formulated with 500 μg aluminum adjuvant (Alhydrogel) and either with 0.02% formaldehyde or without formaldehyde.

[b] Survival ratio of rabbits challenged s.c. on week 6 with spores from the Ames isolate of *B. anthracis*.

[c] Titer expressed as μg of anti-rPA IgG per ml and S.E.M. in parenthesis.

[d] Titer expressed as the reciprocal of the dilution of antiserum at which neutralization of the cytotoxic activity of LeTx on J774A.1 cells was half-maximal (50%; ED_{50}) and S.E.M. in parenthesis. If the TNA assay ED_{50} titer could not be extrapolated from the four-parameter logistic regression curve, the value was arbitrarily assigned a value of '1.0'. The starting dilution for the TNA assay was 1:50.

[e] BLQ, below the limit of quantitation which was 0.072 μg/ml IgG, the concentration of the lowest standard (1.44 ng/ml IgG) multiplied by the lowest starting concentration of the sample (1:50) of the ELISA.

high-level, long-lasting protection in non-human primates [31]. Few studies have been performed on the formulation of rPA vaccines and the effect on the resulting immunological responses in animal models. Aluminum adjuvants are thought to enhance the immune response by localizing the deposition of the antigen, that desorption of antigen can occur in the interstitial fluid, and that both desorbed and adsorbed antigens are processed by antigen-presenting cells [32] and preferentially stimulate the Th2 immune (humoral) response. Anthrax vaccines formulated with either aluminum hydroxide gel or aluminum phosphate adjuvants result in comparable anti-PA titers in humans and guinea pigs [33,30]. Berthold et al. [34] reported a significant increase in ELISA titers to rPA in mice when their vaccine was formulated with either aluminum hydroxide gel or aluminum phosphate adjuvant compared to controls without adjuvant and that ELISA titers to rPA were comparable when either aluminum hydroxide gel or aluminum phosphate adjuvants were used to formulate the rPA vaccine. However, they also found that there was an optimal adjuvant concentration because at higher concentrations of aluminum hydroxide gel adjuvant, the neutralizing antibody titers decreased [34]. We did not observe a decrease in the TNA assay ED_{50} titers in the rabbit animal model at the highest concentration of aluminum tested (500 μg). Both the anti-PA ELISA titer and toxin neutralizing antibody titers have been identified as serological correlates of immunity in rabbits and guinea pigs [13–17,25] and are thus important measurements in developing effective vaccine strategies. Various formulations have been tested in preparing anthrax vaccines based upon rPA for its ability to elicit optimal immunological responses. Recent examples include Toll-like receptor ligands CpG ODN and Resiquimod R-848 [35,36], pluronic F127, a non-ionic, hydrophilic polyoxyethylene-polyoxypropylene block copolymer [37], additional vaccine antigens such as capsule [38] or EF [39,40], DNA vaccines [41], and mucosal vaccine strategies [42,43]. That antibodies have been recognized as important in protection is demonstrated by the number of immunotherapeutic reagents that have been recently suggested [44–49]. However, protection has not always been attributed to toxin-neutralizing antibodies. Brossier et al. [50] proposed that neutralizing anti-PA antibodies may be more important in animal models that are highly susceptible to toxemia than in animal models that are more susceptible to infection. The development of a new rPA vaccine will require the identification of an acceptable aluminum compound, optimal concentration of aluminum, and approved excipients that will enhance the immunological responses necessary for protection against infection in the proper surrogate animal models.

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References

[1] Brey RN. Molecular basis for improved anthrax vaccines. Adv Drug Del Rev 2005;57:1266–92.
[2] Stanley JL, Smith H. The three factors of anthrax toxin: their immunogenicity and lack of demonstrable enzymic activity. J Gen Microbiol 1963;31:329–37.
[3] Mahlandt BG, Klein F, Lincoln RE, Haines BW, Jones Jr WI, Friedman RH. Immunologic studies of anthrax. IV. Evaluation of the immunogenicity of three components of anthrax toxin. J Immunol 1966;96:727–33.
[4] Ivins BE, Welkos SL, Knudson GB, Little SF. Immunization against anthrax with aromatic compound-dependent (Aro^-) mutants of *Bacillus anthracis* and with recombinant strains of *Bacillus subtilis* that produce anthrax protective antigen. Infect Immun 1990;58:303–8.
[5] Ivins BE, Welkos SL, Little SF, Crumrine MH, Nelson GO. Immunization against anthrax with *Bacillus anthracis* protective antigen combined with adjuvants. Infect Immun 1992;60:662–8.
[6] Committee to Assess the Safety and Efficacy of the Anthrax Vaccine MFA. In: Joellenbeck LM, Zwanziger LL, Durch JS, Strom BL, editors. The Anthrax Vaccine: Is It Safe? Does It Work? National Academies Press: Washington, DC; 2002.
[7] Wasserman GM, Grabenstein JD, Pittman PR, Rubertone MV, Gibbs PP, Wang LZ, et al. Analysis of adverse events after anthrax immunization in US Army medical personnel. J Occup Environ Med 2003;45:222–33.
[8] Lange JL, Lesikar SE, Rubertone MV, Brundage JF. Comprehensive systematic surveillance for adverse effects of anthrax vaccine adsorbed, US Armed Forces, 1998–2000. Vaccine 2003;21:1620–8.
[9] Enstone JE, Wale MC, Nguyen-Van-Tam JS, Pearson JC. Adverse medical events in British service personnel following anthrax vaccination. Vaccine 2003;21:1348–54.
[10] Iacono-Connors LC, Welkos SL, Ivins BE, Dalrymple JM. Protection against anthrax with recombinant virus-expressed protective antigen in experimental animals. Infect Immun 1991;59:1961–5.
[11] Pezard C, Weber M, Sirard J-C, Berche P, Mock M. Protective immunity induced by *Bacillus anthracis* toxin-deficient strains. Infect Immun 1995;63:1369–72.
[12] Ivins BE, Pitt MLM, Fellows PF, Farchaus JW, Benner GE, Waag DM, et al. Comparative efficacy of experimental anthrax vaccine candidates against inhalation anthrax in rhesus macaques. Vaccine 1998;16:1141–8.
[13] Williamson ED, Hodgson I, Walker NJ, Topping AW, Duchars MG, Mott JM, et al. Immunogenicity of recombinant protective antigen and efficacy against aerosol challenge with anthrax. Infect Immun 2005;73:5978–87.
[14] Reuveny S, White MD, Adar YY, Kafri Y, Altboum Z, Gozes Y, et al. Search of correlates of protective immunity conferred by anthrax vaccine. Infect Immun 2001;69:2888–93.
[15] Little SF, Ivins BE, Fellows PF, Pitt MLM, Norris SLW, Andrews GP. Defining a serological correlate of protection in rabbits for a recombinant anthrax vaccine. Vaccine 2004;22:422–30.
[16] Weiss S, Kobiler D, Levy H, Marcus H, Pass A, Rothschild N, et al. Immunological correlates for protection against intranasal challenge of *Bacillus anthracis* spores conferred by a protective antigen-based vaccine in rabbits. Infect Immun 2006;74:394–8.
[17] Peachman K, Rao M, Alving CR, Berge R, Leppla SH, Rao VB, et al. Correlation between lethal toxin-neutralizing antibody titers and protection from intranasal challenge with *Bacillus anthracis* Ames strain spores in mice after transcutaneous immunization with

recombinant anthrax protective antigen. Infect Immun 2006;74: 794–7.
[18] Lincoln RE, Fish DC. Anthrax toxin. In: Montie TC, Kadis S, Ajl SJ, editors. Microbial toxins: bacterial protein toxins. New York: Academic Press; 1970. p. 361–414.
[19] Harris-Smith PW, Smith H, Keppie J. Production *in vitro* of the toxin of *Bacillus anthracis* previously recognized *in vivo*. J Gen Microbiol 1958;19:91–103.
[20] Little SF, Ivins BE, Fellows PF, Friedlander AM. Passive protection by polyclonal antibodies against *Bacillus anthracis* infection in guinea pigs. Infect Immun 1997;65:5171–5.
[21] Ivins BE, Welkos SL. Cloning and expression of the *Bacillus anthracis* protective antigen gene in *Bacillus subtilis*. Infect Immun 1986;54:537–42.
[22] Worsham PL, Sowers MR. Isolation of an asporogenic (spoOA) protective antigen-producing strain of *Bacillus anthracis*. Can J Microbiol 1999;45:1–8.
[23] Farchaus JW, Ribot WJ, Jendrek S, Little SF. Fermentation, purification, and characterization of protective antigen from a recombinant, avirulent strain of *Bacillus anthracis*. Appl Environ Microbiol 1998;64: 982–91.
[24] Leppla SH. Production and purification of anthrax toxin. In: Harshman S, editor. Methods of enzymology, vol. 165. Orlando, FL: Academic Press Inc.; 1988. p. 103–16.
[25] Pitt MLM, Little SF, Ivins BE, Fellows P, Barth J, Hewetson J, et al. In vitro correlate of immunty in a rabbit model of inhalational anthrax. Vaccine 2001;19:4768–73.
[26] Ivins B, Fellows P, Pitt L, Estep J, Farchaus J, Friedlander A, et al. Experimental anthrax vaccines: efficacy of adjuvants combined with protective antigen against an aerosol *Bacillus anthracis* spore challenge in guinea pigs. Vaccine 1995;13:1779–84.
[27] Hambleton P, Carman JA, Melling J. Anthrax: the disease in relation to vaccines. Vaccine 1984;2:125–32.
[28] Little SF, Webster WM, Ivins BE, Fellows PF, Norris SL, Andrews GP. Development of an in vitro-based potency assay for anthrax vaccine. Vaccine 2004;22:2843–52.
[29] Little SF, Ivins BE, Webster WM, Fellows PF, Pitt MLM, Norris SLW, et al. Duration of protection of rabbits after vaccination with *Bacillus anthracis* recombinant protective antigen vaccine. Vaccine 2006;24:2530–6.
[30] Turnbull PC, Broster MG, Carman JA, Manchee RJ, Melling J. Development of antibodies to protective antigen and lethal factor components of anthrax toxin in humans and guinea pigs and their relevance to protective immunity. Infect Immun 1986;52:356–63.
[31] Ivins BE, Fellows PF, Pitt MLM, Estep JE, Welkos SL, Worsham PL, et al. Efficacy of a standard human anthrax vaccine against *Bacillus anthracis* aerosol spore challenge in rhesus monkeys. Salisbury Med Bull 1996;87:125–6.
[32] Iyer S, HogenEsch H, Hem SL. Relationship between the degree of antigen adsorption to aluminum hydroxide adjuvant in interstitial fluid and antibody production. Vaccine 2003;21:1219–23.
[33] Turnbull PC, Leppla SH, Broster MG, Quinn CP, Melling J. Antibodies to anthrax toxin in humans and guinea pigs and their relevance to protective immunity. Med Microbiol Immunol Berl 1988;177:293–303.
[34] Berthold I, Pombo M-L, Wagner L, Arciniega JL. Immunogenicity in mice of anthrax recombinant protective antigen in the presence of aluminum adjuvants. Vaccine 2005;23:1993–9.
[35] Klinman DM, Xie H, Little SF, Currie D, Ivins BE. CpG oligonucleotides improve the protective immune response induced by the anthrax vaccination of rhesus macaques. Vaccine 2004;22:2881–6.
[36] Weeratna RD, Makinen SR, McCluskie MJ, Davis HL. TLR agonists as vaccine adjuvants: comparison of CpG ODN and Resiquimod (R-848). Vaccine 2005;23:5263–70.
[37] Coeshott CM, Smithson SL, Verderber E, Samaniego A, Blonder JM, Rosenthal GJ, et al. Pluronic® F127-based systemic vaccine delivery systems. Vaccine 2004;22:2396–405.
[38] Chabot DJ, Scorpio A, Tobery SA, Little SF, Norris SL, Friedlander AM. Anthrax capsule vaccine protects against experimental infection. Vaccine 2004;23:43–7.
[39] Zeng M, Xu Q, Hesek ED, Pichichero ME. N-fragment of edema factor as a candidate antigen for immunization against anthrax. Vaccine 2005;24:662–70.
[40] Duverger A, Jackson RJ, van Ginkel FW, Fischer R, Tafaro A, Leppla SH, et al. *Bacillus anthracis* edema toxin acts as an adjuvant for mucosal immune responses to nasally administered vaccine antigens. J Immunol 2006;176:1776–83.
[41] Hermanson G, Whitlow V, Parker S, Tonsky K, Rusalov D, Ferrari M, et al. A cationic lipid-formulated plasmid DNA vaccine confers sustained antibody-mediated protection against aerosolized anthrax spores. Proc Natl Acad Sci USA 2004;101:13601–6.
[42] Flick-Smith HC, Eyles JE, Hebdon R, Waters EL, Beedham RJ, Stagg TJ, et al. Mucosal or parenteral administration of microsphere-associated *Bacillus anthracis* protective antigen protects against anthrax infection in mice. Infect Immun 2002;70:2022–8.
[43] Mikszta JA, Sullivan VJ, Dean C, Waterston AM, Alarcon JB, Dekker III JP, et al. Protective immunization against inhalational anthrax: A comparison of minimally invasive delivery platforms. J Infect Dis 2005;191:278–88.
[44] Subramanian GM, Cronin PW, Poley G, Weinstein A, Stoughton SM, Zhong J, et al. A phase I study of PAmAb, a fully human monoclonal antibody against *Bacillus anthracis* protective antigen, in healthy volunteers. Clin Infect Dis 2005;41:12–20.
[45] Mabry R, Rani M, Geiger R, Hubbard GB, Carrion Jr R, Brasky K, et al. Passive protection against anthrax by using a high-affinity antitoxin antibody fragment lacking an Fc region. Infect Immun 2005;73:8362–8.
[46] Mohamed N, Clagett M, Li J, Jones S, Pincus S, D'Alia G, et al. A high-affinity monoclonal antibody to anthrax protective antigen passively protects rabbits before and after aerosolized *Bacillus anthracis* spore challenge. Infect Immun 2005;73:795–802.
[47] Peterson JW, Comer JE, Noffsinger DM, Wenglikowski A, Walberg KG, Chatuev BM, et al. Human monoclonal anti-protective antigen antibody completely protects rabbits and is synergistic with ciprofloxacin in protecting mice and guinea pigs against inhalation anthrax. Infect Immun 2006;74:1016–24.
[48] Herrmann JE, Wang S, Zhang C, Panchal RG, Bavari S, Lyons CR, et al. Passive immunotherapy of *Bacillus anthracis* pulmonary infection in mice with antisera produced by DNA immunization. Vaccine 2006;24:5872–80.
[49] Vitale L, Blanset D, Lowy I, O'Neill T, Goldstein J, Little SF, et al. Prophylaxis and therapy of inhalational anthrax by a novel monoclonal antibody to protective antigen that mimics vaccine-induced immunity. Infect Immun 2006;74:5840–7.
[50] Brossier F, Levy M, Mock M. Anthrax spores make an essential contribution to vaccine efficacy. Infect Immun 2002;70:661–4.